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A LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

WITH SUPPLEMENT

BY

OTTO FOLIN

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PREFACE

This manual of biological chemistry for medical students in Harvard Medical School has been revised annually for the past seven years, and it is believed now to meet our needs sufficiently well to warrant publication.

For many years I have been interested in the development of analytical methods applicable to metabolism investigations. The most serviceable of my older methods and some of the newer methods have been taught to our medical students; these are described in the main body of the manual. Others not heretofore included have been incorporated in the supplement, so that nearly all the newer methods devised in the department are now described in this manual.

In connection with the revisions referred to above I am indebted for valuable help to W. R. Bloor, W. Denis, C. J. Farmer, L. J. Morris, F. B. Kingsbury, F. S. Hammett, R. D. Bell, and C. H. Fiske, as well as to my older friend, P. A. Shaffer.

OTTO FOLIN.

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LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

PART I

ACIDIMETRY, ALKALIMETRY, NITROGEN DETERMINATION

Equivalent and Normal Solutions.—Since the molecular weight of sodium hydroxid (NaOH) is 40 and that of hydrochloric acid (HCl) is 36.46, it follows that 40 g. of the former contain the same number of molecules as 36.46 g. of the latter. If 40 g. of sodium hydroxid and 36.46 g. of hydrochloric acid are each dissolved in pure water sufficient to make one liter of solution, each liter will contain the same number of dissolved molecules.

It will take a little less than one liter of water to make a liter of solution because the dissolved substance takes up some space. A normal sodium hydroxid solution contains four per cent. of sodium hydroxid. By per cent. in the case of solutions is usually meant the amount of substance present in 100 c.c. of solution.

Mixing equal volumes of two such solutions is therefore the same as bringing together practically the same number of the two kinds of molecules, and the result is the instantaneous and essentially complete transformation into sodium chlorid (and water).

$$X\ NaOH + X\ HCl = X\ NaCl + X\ H_2O$$

If either or both of the solutions should first be diluted with a considerable bulk of pure water, the result on mixing the two would be the same, for the extra amount of water present takes no part in the reaction (except to the extent of absorbing a part of the heat set free).

The two solutions are equivalent. They also happen to be normal solutions. The hydrochloric acid is normal because it contains 1 g. of active or replaceable hydrogen per liter of solution,

and not because it contains the same number of grams of HCl per liter as there are units in the molecular weight. The sodium hydroxid solution is normal because it is equivalent to a solution containing one gram of replaceable hydrogen per liter.

The molecular weight of sulphuric acid is 98. A sulphuric acid solution containing exactly 98 g. per liter contains therefore the same number of molecules per unit volume as the sodium hydroxid solution containing 40 g. per liter. But one molecule of sulphuric acid requires two molecules of sodium hydroxid for the formation of the neutral salt, sodium sulphate, because the sulphuric acid molecule has two replaceable hydrogen atoms. The solutions are not equivalent, for the sulphuric acid contains 2 g. active hydrogen per liter. It is exactly twice as strong as the sodic hydrate solution; it is a 2 normal solution.

On the basis of the above description of what constitutes a normal solution, calculate the number of grams per liter in tenth normal sulphuric acid (.1N H_2SO_4), fifth normal hydrochloric acid (.2NHCl), half normal oxalic acid (.5N $C_2H_2O_4$, $2H_2O$), fourth normal acetic acid (.25N CH_3COOH), half normal sodic hydrate (.5N NaOH), twentieth normal barium hydrate (.05N $Ba(OH)_2$), fifth normal ammonium hydrate (.2N NH_4OH).

Atomic weights of some of the more important elements: Arsenic (As) 74.96, Barium (Ba) 137.37, Bromin (Br) 79.92, Calcium (Ca) 40.09, Carbon (C) 12, Chlorin (Cl) 35.46, Copper (Cu) 63.57, Hydrogen (H) 1.008, Iodin (I) 126.92, Iron (Fe) 55.85, Lead (Pb) 207.1, Magnesium (Mg) 24.32, Manganese (Mn) 54.93, Mercury (Hg) 200.6, Nitrogen (N) 14.01, Oxygen (O) 16, Phosphorus (P) 31.04, Potassium (K) 39.1, Sulphur (S) 32.07, Tin (Sn) 119, Tungsten (Wo) 184, Uranium (U) 238.5, Zinc (Zn) 65.37.

The same description of normal solutions applies to other substances than acids and alkalis, as for example, reducing and oxidizing substances such as potassium permanganate, potassium bichromate, iodin, cupric hydrate, stannous chlorid. A normal solution is here one capable of liberating 1 g. of reducing hydrogen (or of giving off exactly sufficient oxygen to oxidize one gram of hydrogen) per liter. Potassium permanganate, for example, in the presence of sulphuric acid and some easily oxidizable substance is decomposed as follows:

$$2KMnO_4 + 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 3H_2O + 5O$$
$$+ 2MnSO_4$$

As the two permanganate molecules liberate oxygen enough for ten hydrogen atoms it takes only one fiftieth of the molecular weight expressed in grams (3.156 g.) to make one liter of tenth normal solution.

The calculation of what constitutes normal or equivalent solutions of any reagent is not very difficult provided the equation representing the chemical reaction involved is thoroughly clear.

To determine whether a given unknown solution is acid or alkaline it is usually sufficient to dip a piece of delicate violet colored litmus paper into it. (If the solution is acid the test paper turns red; if alkaline it turns blue.) Litmus, the substance with which the paper has been impregnated, is a complex organic product, and is one of the most familiar representatives of a most useful class of organic compounds which are so sensitive to acids or alkalis, or both, that they clearly and unmistakably indicate the presence of free acid or alkali even when the amounts present are so small as to be practically unweighable. By means of such *indicators* and accurate measuring instruments (measuring flasks, burets, and pipets), it becomes a simple matter to determine (by titration) the relative concentration or equivalence of acid and alkaline solutions. By their help it is possible to prepare with very little labor normal or tenth normal solutions, even of acids or alkalis which cannot be weighed on the balance, as for example, hydrochloric acid and ammonia, both of which are gases. Before this can be done we must, however, possess one normal or standard solution prepared from some substance which can be weighed.

Volumetric analysis consists of measuring the value of an unknown solution in terms of another the value of which is known (titration). The known solution is prepared directly or indirectly by the help of the analytical balance, and the first step in any kind of volumetric analysis is the preparation of the standard solution by means of which the values of others are to be determined.

Every student who has had no experience in the use of the analytical balance must consult the instructor before proceeding. He should also ask for instruction as to the proper use of measuring flasks, pipets, and burets before using them. He must particularly learn when the presence of unmeasured quantities of water does not interfere with the accuracy of the work and when a single drop of

unmeasured water introduces a perceptible error. (See Sutton's Volumetric Analysis, Part 1—"Instruments and Apparatus.")

All the common mineral acids and strong alkalis contain so much water that it is in practice not feasible to weigh out with sufficient accuracy the theoretical quantity required for a standard solution of acid or alkali. The carbonates of sodium or calcium (or the carbonates of sodium or potassium, obtained by ignition of the corresponding oxalates) give exceedingly accurate results. Oxalic acid is very serviceable as starting material for the preparation of standardized solutions of acids and alkalis if it is pure and has lost none of its water of crystallization.

1. Preparation of .5N Oxalic Acid (500 c.c.) — The usefulness of oxalic acid as a starting point for the preparation of standard acids and alkalis is due entirely to the fact that it can be obtained chemically pure and in condition suitable for direct weighing. Oxalic acid is, however, not a strong enough acid to titrate well with all the common indicators, and it is therefore not serviceable for acidimetric titrations in general. But by means of oxalic acid and with phenolphthalein as indicator, standard solutions of a strong alkali, like caustic soda, can be obtained, and by means of the latter standard solutions of the stronger mineral acids can then be prepared.

The reason why the strong acids and alkalis give more accurate and reliable results is the fact that the salts which they form when neutralized are not appreciably hydrolyzed by water into acid and base, as are the corresponding salts of the weaker acids and bases. The zone of neutrality to different indicators is therefore more sharply defined, and corresponds more nearly to the point represented by the presence of exactly equivalent amounts of acid and alkali.

Weigh accurately (to the fourth decimal) a small, clean, and dry beaker or large crucible. Then add to the weights on the balance pan 15.7560 g., and add oxalic acid to the vessel on the other side until exact equilibrium is reached. Dissolve in distilled water this oxalic acid without the loss of a single crystal. The acid dissolves rather slowly. The solution is therefore best made in a beaker by the aid of gentle heating with about 250 c.c. water. Transfer every drop of the solution to a measuring flask (500 c.c.), carefully rinsing the last traces from the beaker into the flask by means of successive small amounts of cold distilled water. Cool the flask in running tap water until the

contents of the flask have reached the room temperature. (If a thermometer is used it must be rinsed carefully before it is removed from the flask.) Fill up with water until the lower side of the "meniscus" is exactly even with the 500 c.c. mark. Stopper the flask, and invert several times (30-40) so that the solution is thoroughly mixed. Transfer to a clean, *dry* bottle; label and preserve.

Using a strong base like sodium hydroxid and a sensitive indicator like phenolphthalein for the titration, it is possible to obtain quite reliable and accurate results with oxalic acid. The volumetric determinations involved in metabolism studies and urine analysis are, however, extensively based on titrating ammonia, which is a very weak base. Phenolphthalein, because of its high degree of sensitiveness to weak acids and its lack of sensitiveness to weak bases, is useless in titrations of ammonia. The oxalic acid and the phenolphthalein are therefore used only for the purpose of securing a standard alkali solution.

2. Preparation of .5N Sodic Hydrate (1,000 c.c.).—The sodic hydrate used for standard solutions must be as free as possible from carbonates, because otherwise the solutions will not have the same titrating value with all the common indicators. Sodic hydrate absorbs rapidly carbonic acid from the atmosphere. The carbonic acid should first be removed from the alkali, and the solutions must afterwards be protected from too much exposure to the carbonic acid of the air. As the carbonates are almost insoluble in very strong sodic hydrate solutions, 40 per cent. solutions in which the carbonates have settled can be used as a starting point.

Transfer about 80 c.c. clear 40 per cent. sodic hydrate solution to a large flask or bottle, and add 1,000-1,200 c.c. water. Dissolve about 2 g. barium hydroxid in about 100 c.c. hot water, and without filtering pour this solution into the sodic hydrate solution. Cover with a watch glass, and set aside over night. By means of a siphon or compressed air remove the clear, supernatant solution to another flask.

To determine the exact value of the solution it is only necessary to find out how much of it is required for the neutralization of a known volume of the .5N oxalic acid solution. With a dry and clean pipet transfer 25 c.c. of the oxalic acid into a beaker or flask. Dilute it by adding 100-150 c.c. of water, and add two

drops of the indicator (1 per cent. alcoholic solution of phenolphthalein).

Fill a dry, clean buret with the alkali and cover with a test tube. After adjusting the solution in the buret to the zero mark, run it into the diluted oxalic acid, more and more cautiously toward the end until finally one single drop produces a deep red coloration. Note the volume of alkali required (to within .05 c.c.). Repeat the titration until two successive ones give exactly the same figure.

In titrating acids and alkalis the alkali must always be run into the acid solution, not vice versa.

From the result obtained calculate how much of the alkali would be required to neutralize one liter of the oxalic acid solution. By means of a 100 c.c. pipet and a buret, transfer the required amount of the alkali to the liter measuring flask, and fill up to the mark with water. Mix, transfer to a dry bottle, label, and stopper with a rubber stopper.

As a check on the work determine the concentration of an unknown hydrochloric acid solution (furnished), using as indicator (a) phenolphthalein (b) alizarin red (2 drops 1 per cent. aqueous solution).

3. Half Normal Hydrochloric Acid.—Concentrated hydrochloric acid is approximately 10 N solution of HCl. From it prepare 1,000 c.c. .5N solution, using the half normal sodium hydroxid as a standard and alizarin red as indicator. The most convenient way is first to prepare 1,200-1,300 c.c. of a solution somewhat stronger than half normal, and then, on the basis of titrations with the standard alkali, to dilute the required amount with water to one liter. One liter of half normal hydrochloric acid is enough for all the analytical work described in this manual.

Those who have had experience in volumetric analysis may use simply standardized solutions instead of the half normal in the case of hydrochloric acid and in the case of all other standard solutions, but beginners should not omit the preparation of a strictly half normal acid.

4. Tenth Normal Acid and Alkali.—From the half normal stock solutions of hydrochloric acid and sodium hydroxid, pre-

pare 1,000 c.c. .1N hydrochloric acid and .1N sodium hydroxid. The solutions so obtained should be equivalent.

5. Strong and Weak Acids; the Use of Different Indicators.* (A) Titrate 25 c.c. tenth normal hydrochloric acid (+ 100 c.c. water) with the tenth normal alkali, using as indicator (a) phenolphthalein (b) methyl orange (c) alizarin red. Repeat the above mentioned three titrations in the presence of 10 c.c. ammonium chlorid solution (2 per cent.). Repeat the titration with each indicator using in place of the hydrochloric acid (a) 25 c.c. .1N phosphoric acid (b) 25 c.c. .1N lactic acid.

Record the titrations in tabular form:

	Phenolphthalein: c.c.—End point.†	Methyl orange: c.c.—End point.†	Alizarin red: c.c.—End point.†
HCl:			
HCl NH_4Cl:			
Phosphoric acid:			
Lactic acid:			

(B) Dilute (a) 20 c.c. half normal hydrochloric acid (b) 20 c.c. half normal lactic acid (supplied) to 500 c.c., making approximately .02 N solutions. (Measuring cylinders are accurate enough for the dilutions referred to here.)

From each .02 normal solution prepare four 250 c.c. portions of more dilute acids, namely: .005 N; .001 N; .0002 N; .0001 N. Arrange in a labeled series ten beakers, flasks, or (small, white) bottles, similar in size and shape, and transfer to each one an approximately equal volume (50 c.c.) of one of the acid solutions. Add to each acid in the series one drop (no more) of "Töpfer's Reagent" (.5 per cent. dimethylamino-azo-benzol solution in alcohol). For comparison add one drop of the indicator to 50 c.c. water. Test the acidity of each diluted acid with (a) congo red paper (b) litmus paper. Also taste each solution. (Very dilute acids are best tasted by "rinsing the mouth" first with distilled water and then with a liberal quantity of the acid.)

---

* Consult Sutton's Volumetric Analysis—"Indicators."

† End point may be designated as sharp, fair, indistinct, or indeterminate.

Record the results of the tests in the tabular spaces indicated below:

	Töpfer's Reagent * HCl—Lactic	Congo Red * HCl—Lactic	Litmus * HCl—Lactic	Sour Taste * HCl—Lactic
Acid:				
.02 N				
.005 N				
.001 N				
.0002 N				
.0001 N				

6. Special Test for Hydrochloric Acid.—Gunzberg's reagent (2 g. phloroglucin and 1 g. vanillin in 100 c.c. alcohol) is very reliable as a means of distinguishing between hydrochloric acid and lactic or other organic acids. The reaction is best carried out as follows:

Transfer 5-6 drops of the reagent to a shallow evaporating dish, and evaporate to dryness over a water bath consisting simply of a beaker of boiling water. The alcoholic solution spreads all over the dish, leaving a thin coating of the dry reagent. By means of pipets, or glass tubes drawn out like pipets, transfer one drop of .01 N hydrochloric acid to one side of the dish, and on another side deposit one drop of .1 N lactic acid, and again place the dish on the water bath. A purplish ring is quickly formed around the hydrochloric acid drop while the lactic acid remains colorless.

Heating over the flame may be substituted for the water bath, but the least overheating tends to obscure the reaction by charring. This reaction is extensively used in the examination of stomach contents.

7. Special Test for Lactic Acid.—More or less specific tests for lactic acid are known and are considered important because of the frequency with which lactic acid is found in the stomach contents of those suffering from carcinoma of the stomach. A convenient yet reliable method is the following:

* The reaction may be designated as follows: ++++, +++, ++,+, +—, —.

To 5-10 c.c. of .1 N lactic acid (or filtered stomach juice) in a large test tube add a few drops (.5 c.c.) of normal hydrochloric acid and about 10 c.c. of ether. By cautiously inverting the test tube during 3-4 minutes (taking care to avoid explosions due to expanding ether vapors) the lactic acid is in part taken up by the ether. By means of a 25 c.c. or 50 c.c. pipet and suction, remove the lower aqueous layer as completely as possible. Decant the remaining ether into another test tube so as to free it from the few drops of aqueous solution not taken out by the pipet. Then add to the ether solution .2 per cent. ferric chlorid solution,* a little at a time with shaking, until the maximum yellow color is obtained. The amount of solution added and the depth of the color obtained give a rough index as to the amount of lactic acid present.

8. Nitrogen Determination in Ammonium Salts.—The most convenient and useful analysis of nitrogenous products of physiological significance is the determination of the nitrogen. The nitrogen of such products can be split off by hydrolysis in the form of ammonia, which can then be determined by distillation and subsequent titration.

In a small beaker weigh (to the fourth decimal) 3-3.5 g. pure ammonium sulphate. The salt contains traces of water (.5-1 per cent.), unless it has been dried by heating 1-2 hours at about 110° C.; it should be kept in a desiccator over sulphuric acid. Dissolve the salt without the loss of a single crystal in a 500 c.c. volumetric flask, add 1-2 c.c. concentrated hydrochloric acid, and fill up to the mark with water. The acid is added to keep out moulds. Mix thoroughly and transfer to a dry bottle, or to a bottle freshly rinsed twice with about 25 c.c. of the solution.

This solution should be stoppered, labeled, and preserved as a standard solution. It is used to check up the accuracy of ammonia determinations and sulphate determinations, and later for colorimetric nitrogen determinations.

By means of a pipet measure 25 c.c. of the ammonium sulphate solution into each of two Jena flasks (capacity about 1 liter), and add about 350 c.c. of water, a pinch of talcum powder,

* 10 c.c. of 10 per cent. ferric chlorid solution in 400-500 c.c. of tap water.

and 5 c.c. of saturated sodium hydroxid solution. The talcum powder is added to prevent bumping. The alkali is added in excess to set free the ammonia. In order to avoid loss of the volatile ammonia the alkali must not be added until everything is ready for the distillation.

Beginners should always consult the instructor before adding the alkali.

The lower part of the condenser must dip into receivers. The receivers are flasks (capacity, about 750 c.c.). In many laboratories milk bottles are used as receivers. The receivers should contain 25 c.c. .1N acid and two or three drops of indicator (alizarin red).

The indicator is added so that if by any chance the ammonia distilled over is more than enough to neutralize the acid that fact is at once revealed. When this happens another 10 or 25 c.c. of .1 N acid should be added.

Immediately after adding the alkali the flask should be connected with the condenser. The flame for the boiling should then be applied without delay. The distillation should be continued until the distillate no longer shows the faintest alkaline reaction when dropped on sensitive litmus paper. During the last few minutes the delivery tube should not dip below the surface of the liquid in the receiver. The time required for the distillation is usually about half an hour from the time the liquid begins to boil. If a great deal of water is added to the ammonium salt solution, it usually takes a little longer to distill off all the ammonia. Brisk boiling is desirable.

Titrate the remaining uncombined hydrochloric acid in the receiver, and from the figure obtained calculate the amount of nitrogen recovered (in milligrams), and compare with the theoretical figure which the amount of ammonium sulphate taken should give.

In calculating the nitrogen from the titration figures, the amount of acid combined with the ammonia can be regarded as a tenth normal nitrogen solution, each cubic centimeter of which accordingly represents 1.4, or more accurately 1.401, milligram nitrogen. Example: 25 c.c. .1 N HCl was the original amount of acid in the receiver. After the distillation the titration of the distillate re-

quired 2.1 c.c. of .1N NaOH. The ammonia had therefore neutralized 25 — 2.1 or 22.9 c.c. of the tenth normal acid, 22.9 × 1.4 = 32.06 (milligram nitrogen).

9. Kjeldahl's Method for Determining Nitrogen.—In a 100 c.c. volumetric flask dissolve 1-1.5 g. accurately weighed urea. Add a few drops (.5-1 c.c.) concentrated hydrochloric acid, and make the volume up to 100 c.c., mix, and transfer to a clean and dry bottle, label, and preserve.

Pipet 5 c.c. of this solution into each of two Kjeldahl flasks, add 15 c.c. concentrated ammonia free sulphuric acid and 2 c.c. 5 per cent. copper sulphate solution, and boil 30-40 minutes.

When organic substances are boiled with strong sulphuric acid both oxidation and hydrolysis take place. The oxidation occurs at the expense of the oxygen in the sulphuric acid, and the latter is consequently reduced. The sulphurous fumes thus produced are very irritating to the mucous membranes of the nose and throat. The digestion must, therefore, be made in a hood having a reasonably good draft.

Instead of a hood a "fume absorber" can be used. By the help of an ordinary water pump (of glass) the fumes are then partly aspirated directly into the drain pipes, and the remainder is collected in the lower part of the fume absorber.

When the urea is decomposed by means of boiling sulphuric acid, it is simply hydrolyzed into carbonic acid and ammonia, and the ammonia combines with the sulphuric acid to give ammonium sulphate. From this point on, the nitrogen determination is essentially the same as the nitrogen determination of ammonium sulphate described above. There are, however, one or two points to be noted. Since the ammonium sulphate is now accompanied by a relatively enormous amount of surplus sulphuric acid, a great deal of alkali must be added to set free the ammonia. (The amount of alkali required for this purpose should be determined by a rough titration of 15 c.c. concentrated sulphuric acid dissolved in 500-700 c.c. tap water—the saturated sodium hydroxid solution being added with a measuring cylinder. Rather more alkali than is indicated by the titration should be used.) Before adding this alkali the sulphuric acid digest must, of course, be cooled and diluted (with about 300 c.c. of water).

Practices differ somewhat with regard to the details in the dis-

tillation of Kjeldahl digestion mixtures. Some use large Kjeldahl flasks for the digestion (500-750 c.c.), and then use the same flasks for the distillation. Others use small Kjeldahl flasks (200 c.c.), and pour the cooled acid into liter flasks containing about 200 c.c. of water, rinsing the last traces of acid into the flasks by three or four successive small quantities of water. The latter procedure has this advantage, that there is no danger from explosions because the acid is poured into water, and not vice versa. Whichever procedure is used, it must not be forgotten that much heat is generated when concentrated sulphuric acid and water are mixed, and still more when so much acid is suddenly neutralized by the addition of the strong alkali. The desirability of working understandingly and with reasonable dispatch when adding the alkali and starting the distillation is clear. The alkali and acid should not be mixed by shaking until after the flask is attached to the condenser.

The distillate is collected in 25 c.c. .1 N acid and 50 c.c. water, and the nitrogen recovered is determined and compared with the theoretical figure as in the ammonium sulphate analysis.

10. Determination of Nitrogen in Uric Acid.—Transfer 50-70 mg. of pure uric acid to a clean, dry test tube. Weigh the test tube and uric acid (to the fourth decimal). Shake most of the uric acid into a dry Kjeldahl flask, and again weigh accurately the empty test tube. The difference between the two weighings is the amount of uric acid taken. In the same way, charge another dry Kjeldahl flask with 50-60 mg. of uric acid. To each flask add 15 c.c. of concentrated sulphuric acid and 2 c.c. copper sulphate solution, digest for 30-40 minutes, distil, and determine the ammonia. Calculate as nitrogen, and compare with the theoretical figures.

PART II

CATALYSIS, CATALYZERS, FERMENTS *

1. **Hydrogen-ion.**—In each of two test tubes place about 5 c.c. of 2 per cent. cane sugar solution. To the solution in one of the test tubes add a few drops of concentrated hydrochloric acid. Boil the contents of both for five minutes, or heat in a beaker of boiling water for ten minutes. Cool. To the contents in each test tube add a few drops of 10 per cent. copper sulphate solution and a little saturated sodic hydrate solution (2 c.c.). Heat the alkaline contents almost to boiling. Note and explain the result. (Cane sugar when split by hydrolysis yields reducing sugars.)

2. **Hydroxyl-ion.**—Fill a small test tube up to within about 1 c. from the top with 1 per cent. tannic acid solution. Add a few drops sodic hydrate solution, mix quickly, and let stand for a few minutes.

3. **Metallic salts.**—To 5 c.c. of urine in each of two Kjeldahl flasks add 5 c.c. concentrated sulphuric acid. To one add a small crystal of copper sulphate. Heat to gentle boiling for 10 minutes. Compare the rate of disappearance of the brown color.

4. **Pepsin.**—Prepare a pepsin solution from the mucous membrane of a pig's stomach as follows: Strip off the mucous membrane from a pig's stomach, mix with 300 c.c. of approximately decinormal hydrochloric acid (the "concentrated hydrochloric acid" is approximately a 10 N solution) in a wide-mouth bottle (capacity 900-1,000 c.c.), and let stand over night. Remove by decantation 75 c.c. of the stomach extract. To the remaining mixture in the bottle add 5-10 c.c. of chloroform, cork tightly, shake vigorously for a few seconds, label, and place in an incu-

* See Hammarsten: "Catalysis" and "catalytic reactions."

bator. The stomach will digest itself and give a solution suitable for the later study of peptones.

Suspend a piece of egg albumin, or a Mett tube, in the top of each of the following solutions:

(a) 5 c.c. of undiluted gastric extract,

(b) 5 c.c. consisting of equal volumes of the juice and .1 N hydrochloric acid,

(c) 5 c.c. consisting of one part of juice to 3 parts .1 N hydrochloric acid,

(d) 5 c.c. of juice diluted as in (b) and heated in a water bath at a temperature of 75° for 15 minutes.

Put all the solutions in an incubator (the warm room) over night. Note the results and explain.* Compare results with those of other students.

To 20 c.c. of the pepsin solution add half a volume of disodic phosphate solution (10 per cent.) and half a volume of calcium acetate solution (10 per cent.). Filter off the precipitate and wash once with water. Dissolve the precipitate in a minimum quantity of half normal hydrochloric acid. Dialyze over night. Remove the liquid from the dialyzing tube, test its reaction with congo red, and dilute with water or with dilute HCl (which?) to the volume of the pepsin solution originally taken (20 c.c.). With this solution repeat (a) and (b).

5. Trypsin.†—Free a beef pancreas from fat, cut up fine, and weigh; transfer to wide-mouth bottle and add 3 c.c. 10 per cent. alcohol for each gram of pancreas. Add 5 c.c. of chloroform, cork tightly, shake, and set aside for two or three days.

Then take out 25-35 c.c. of the clear liquid and pour on a filter. Stopper tightly again, and put the bottle in the warm room (or incubator) to be preserved for later experiments on "amino acids." With the filtered portion of the pancreatic extract make the two following experiments:

(a) Test its digestive power on egg albumin (Mett's tubes),

(b) Determine the effect of heat (75° for 15 minutes) on the digestive power of the trypsin solution.

6. Malt Diastase.—Heat 10 g. of dry malt in a dry test tube to 100° for 15 minutes (in boiling water bath). The test tube

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* See Hammarsten: "Pepsin"—"Pepsin Test."

† See Hammarsten: "Trypsin."

must be submerged to as great a depth as the height of the malt within the tube. Digest with 100 c.c. water over night at room temperature. Filter.

Digest 10 g. of unheated malt with 100 c.c. water over night as before. Filter.

With the malt filtrates and a thin boiled starch paste (1 g. starch to 100 c.c. water) make the following experiments:

Mix 5 c.c. of the starch paste with 15 c.c. of each malt extract, and let stand at 75-80° (in a beaker of water).

In each case determine approximately when all the starch has disappeared. To do this, transfer at five-minute intervals a few drops of the digestive fluid, by means of a glass tube, from the test tube into a porcelain evaporating dish, and add drop by drop dilute iodin solution (Iodin Test).

Heat 5 c.c. of a diastase solution to boiling, cool, and test (at 75-80° C.) its power to digest starch.

7. **Reversible Reactions (Mass Law).***—Mix 5 c.c. methyl acetate, 100 c.c. water, and 1 drop concentrated sulphuric acid in a small flask (200-300 c.c.).

(a) Titrate 5 c.c.

(b) Boil for about 5 minutes, using reflux condenser. Cool, remove 5 c.c., titrate the acidity (what indicator?), and calculate the acidity for 100 c.c.

Continue the boiling for one hour, and repeat the titration.

(c) Mix 5 c.c. of glacial acetic acid with 100 c.c. of methyl alcohol, and add one drop of concentrated sulphuric acid. Remove 5 c.c., dilute this with water, and titrate the acidity. Introduce the preparation into a 250 c.c. flask attached to a reflux condenser, and boil for two hours. Cool, remove 5 c.c., dilute, and titrate as before.

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* See Hammarsten.

PART III

FATS

1. Solubility of Fats.—Test the solubility of tallow in water, 5 per cent. NaOH, ether, chloroform, and alcohol, carefully avoiding the vicinity of a flame.

Let a drop of the ether solution fall on paper, and note the result.

Dissolve in 3 c.c. of warm benzol enough tallow to give a moderate precipitate on cooling. Place some of the precipitate on a slide under a cover glass, examine, and describe. Note especially the shape of the ends of the individual crystals.

2. "Iodin Number." Degree of Unsaturation of Fats; Wijs' Method.—Start simultaneously the determination of the iodin number of cottonseed oil and beef tallow. Weigh about .3 g. of cottonseed oil, or about 1 g. of beef tallow, into a 250 c.c. flask, and dissolve in chloroform (10 c.c.). Add 25 c.c. Wijs' iodin solution with a pipet, stopper, and put in a dark place for half an hour. Add 15 c.c. of 10 per cent. potassium iodid, and dilute with 100 c.c. of water, titrate the excess of iodin (partly in solution in the water, partly in the chloroform) with .1 N sodium thiosulphate, by running the latter into the flask until, after repeated shaking, both the chloroform and the watery solution are but faintly straw colored. Then add a few drops of a 1 per cent. starch solution, and continue the titration to the disappearance of the blue color.

While waiting for absorption to take place, the value of the iodin solution may be determined in terms of .1 N thiosulphate by adding KI and titrating in the same way as above. The difference between the two values represents the amount of iodin absorbed by the fat, and is calculated in grams of iodin per 100 g. of fat.

Example:

.3 g. cottonseed oil, when treated as above, required 35 c.c. of .1 N thiosulphate for back titration.

25 c.c. of the iodin solution required 60 c.c. of .1 N thiosulphate.

The oil therefore absorbed iodin corresponding to 60 — 35 = 25 c.c. .1 N thiosulphate, i.e., 25 c.c. .1 N iodin or 25 × .0127 g. Iodin = .317 g. I. The Iodin Number is, therefore, $\frac{100}{3}$ × .317 = 105.6.

Wijs' Iodin Solution.—Dissolve 13 g. of iodin in 1 liter of glacial acetic acid. Titrate the iodin content of the solution, and then pass washed and dried chlorin gas into the solution until the titration number is doubled. A very distinct change in the color of the solution indicates when this has taken place.

The thiosulphate solution is prepared by dissolving 24 g. of the crystallized salt in 1 liter of water and standardizing it in the usual way (see page 127).

3. Saponification and Preparation of Fatty Acids.—Boil about 20 g. of beef tallow with 100 c.c. of alcoholic solution of sodium hydrate on a water bath over night, or until the residue is dry. To the mixture add about 300 c.c. water and heat to boiling. To the hot solution add a few drops of methyl orange; while continuing the heating (and stirring), acidify with dilute sulphuric acid, and filter. Save the filtrate which contains glycerin, then wash the fatty acid residue several times with hot water. Throw away the washings.

Transfer the "glycerin filtrate" to an evaporating dish, label, and place on the water bath for evaporation to dryness.

4. Solubility of Fatty Acids.—Test the solubility of the fatty acid mixture prepared from tallow in water, 5 per cent. NaOH, ether, alcohol, and benzol. Compare the results with those obtained with fat.

Let a drop of the ether solution fall on paper, and note the result.

Dissolve enough of the fatty acid mixture in warm alcohol to give moderate precipitate on cooling. Examine the crystals under the microscope, and describe as in the case of the fat crystals.

5. Spontaneous Saponification of Fats.—Dissolve about .5 g. tallow, or a few drops of oil, in 10 c.c. warm alcohol in a test

tube (avoid fire!). Add 3-4 drops phenolphthalein solution, and then, drop by drop, tenth normal sodic hydrate solution (alcoholic sodic hydrate solution is best) until the indicator reveals a distinctly alkaline reaction. Let the mixture stand in a warm room over night, and again add alkali (drop by drop) until the alkaline reaction reappears.

6. Titration of Higher Fatty Acids.—Dissolve about .2 g. fatty acid mixture in 10 c.c. warm alcohol or benzol (avoid fire!). Add 3-4 drops phenolphthalein, and titrate with tenth normal alcoholic sodic hydrate solution until an alkaline reaction is obtained. One cubic centimeter of the alkali corresponds to how much fatty acid?

On the basis of the solubilities and reactions of fats, fatty acids, and soaps, work out a scheme for their separation and identification. Apply the scheme to three unknowns furnished.

7. Fat Digestion with Lipase (Castor Bean).—Remove the shells from 10 g. fresh castor beans, break them up as fine as possible, and allow to stand over night in a loosely stoppered test tube full of alcohol ether mixture. Pour off, grind the beans to a powder in a small mortar, transfer to a test tube, and let stand under ether over night. Filter with suction, and wash two or three times with small amounts of the alcohol ether mixture. Grind with the powder in the order named, 5 c.c. .1 N sulphuric acid (supplied), 5 c.c. of neutral cotton oil (Sp. gr. .92), and 5 c.c. lukewarm water. The water should be added a little at a time and thoroughly worked into the mixture so that at the end of the operation a good emulsion is secured. Cover the evaporating dish, and let stand in a warm place over night.

Add 50 c.c. of alcohol, 10 c.c. of ether, and a few drops of phenolphthalein, and titrate with .5 N sodium hydrate. Calculate the amount of fatty acid and the per cent. of fat digested.

8. Glycerin and the Acrolein Test.—To about 5 g. acid potassium sulphate ($KHSO_4$) in a porcelain crucible add one drop of glycerin, heat over a direct flame, and note the pungent odor and tear-begetting quality of the fumes. Note how much heat must be applied to secure an unmistakable test.

When the filtrate (saved from the saponification of beef tallow) has evaporated to dryness, the residue obtained is sodium sul-

phate; mixed with it there should be some glycerin. (How much glycerin might be there?)

Mix with a glass rod 2-3 g. of this residue with 5-6 drops concentrated sulphuric acid in a dry crucible, and apply heat. If an unmistakable acrolein test is not obtained, repeat with more of the residue.

9. Emulsification.—Put 1-2 c.c. of a solution of sodium carbonate (.2 per cent.) in a watch glass, and place in the center a drop of rancid oil. The oil soon shows a white rim, and a milky opacity spreads over the solution. Note with the microscope the active movements in the vicinity of the fat drop, due to the separation of minute particles of oil.

Examine a sample of milk under the microscope. The fat should be in a state of fine emulsion.

10. Lecithin.—Demonstrate myelin movements (observed on mixing lecithin with water).

Mix a small piece of lecithin in water in a test tube. Shake vigorously for a time, and state what occurs. To the contents add concentrated caustic soda and boil. Note the fishy odor of trimethylamine (from the cholin). Acidify the solution—fatty acids are precipitated, and glycerophosphoric acid is left in solution. Write the graphic formula for lecithin.

11. Cholesterin; Liebermann's Reaction.—Dissolve a crystal of cholesterin in 10 c.c. of dry chloroform, and to this solution add a few drops of acetic anhydrid (formula?) and one drop of concentrated H_2SO_4. Shake. The liquid becomes rose red, blue, then dark green.

PART IV

CARBOHYDRATES

One of the marked characteristic properties of the monosaccharids is the ease with which under certain conditions they abstract oxygen from the hydroxids of the heavy metals, setting free either the metals or their lower oxids. Most of the reduction tests for "sugar" are based on this fact. Because the occurrence of sugar in urine is of clinical importance and because it is sometimes difficult to determine with certainty whether a given urine does or does not contain abnormal amounts of sugar, a great many reduction tests, with several modifications of each, have been proposed—all designed to facilitate the detection of abnormal amounts of sugar in human urine.

1. Trommer's Test for Sugar.—Trommer's test represents the oldest and simplest of the many methods of testing for sugar by means of its ability to reduce cupric hydroxid to (greenish yellow, yellow, or reddish) cuprous oxid.

To about 5 c.c. of one per cent. dextrose solution in a test tube add 3 c.c. ten per cent. sodic hydrate solution (or 1 c.c. of a saturated solution), and then add drop by drop, with shaking, one per cent. copper sulphate solution (or saturated copper acetate solution) so long as the copper hydroxid formed readily dissolves on shaking. Heat the solution, or the upper part of it, nearly to boiling, and note the character of the ensuing reduction.

In the case of urines with traces of sugar, Trommer's test may fail because too much copper (yielding black copper oxid) or too little (yielding no precipitate) was used. To obviate these difficulties there have been proposed many different cupric hydroxid reagents in which the surplus cupric hydroxid is kept in solution instead of being precipitated.

Bertrand

2. Fehling's Test.—Fehling's solution, which in the past has been extensively used for the quantitative determination of sugar, is made up as follows:

SOLUTION 1. Copper sulphate solution. Weigh out accurately 17.325 g. purest obtainable copper sulphate. Dissolve in hot water, cool, and make up to a volume of 500 c.c.

SOLUTION 2. Alkaline tartrate solution. Weigh (on rough scales) 63 g. potassium hydroxid and 100 g. Rochelle salt (sodium potassium tartrate), transfer to a beaker, add water, stir until dissolved, and dilute to a volume of 500 c.c.

The two Fehling's solutions must be kept in separate and well-stoppered bottles (particularly the alkaline tartrate solution).

When used for quantitative sugar determinations, the copper sulphate solution must be measured out with pipets as carefully as any other standard solution, but the alkaline tartrate solution can be measured out with cylinders. Each c.c. of the copper solution corresponds to 5 mg. of dextrose, 6.7 mg. of lactose, 7.4 mg. of maltose, or 4.75 mg. of "invert sugar," when completely reduced.

The two component parts of Fehling's solution are kept in separate bottles, because when mixed the solution gradually deteriorates. For qualitative tests it is, however, perfectly safe and more convenient to mix enough of the solutions for a day's tests. To 10 or 20 c.c. of each solution mixed in a flask add two volumes (40 or 80 c.c.) of water and shake. With this diluted Fehling's solution the test for sugar is made as follows:

Pour 5-10 c.c. of Fehling's solution into a test tube and heat to boiling (the solution must remain clear). Now add one or two c.c. of sugar solution, or of urine, and again heat to boiling. If more than minute traces of sugar are present, cuprous oxid is precipitated. The mere fading of the solution does not indicate sugar in urine.

3. Benedict's Test.—One of the best qualitative tests for sugar in urine by means of copper solutions is the one recently proposed by S. R. Benedict. Benedict's reagent is so adjusted that it is rather more sensitive to dextrose than Fehling's solution, yet is not reduced by creatinin or uric acid, and little, if at all, by chloroform (which is often added as a preservative to urine). Unlike Fehling's reagent it consists of a single solution. The reagent is made as follows:

Dissolve 85 g. sodium citrate and 50 g. anhydrous sodic carbonate in 400 c.c. of water. Dissolve 8.5 g. copper sulphate in 50 c.c. of hot water. Pour the copper sulphate solution slowly, and with stirring, into the alkaline citrate solution. Filter if necessary. Label and preserve.

Heat to boiling about 5 c.c. of Benedict's reagent in a test tube together with a pebble or two, to prevent bumping. Add about 1 c.c. of sugar solution (or urine) and boil for 1-2 minutes. If more than two- or three-tenths per cent. of sugar is present, the solution will be filled with a colloidal (greenish, yellow, or reddish) precipitate. With smaller amounts of sugar the precipitate will usually appear only on cooling (the cooling should not be hastened by immersion in cold water).

4. Reduction Test for Sugar in Normal Urine.—That most human urines contain distinct traces of reducing sugar can be shown by the use of more sensitive copper reagents. One such reagent can be made as follows:

(A) Dissolve 5 g. of crystallized copper sulphate in 100 c.c. of hot water, and to the cooled solution add 60-70 c.c. of pure glycerin.

(B) Dissolve 125 g. of anhydrous potassium carbonate (with stirring) in 400 c.c. water.

Mix one volume of the glycerin-copper solution (A) with two volumes of the potassium carbonate solution (B). Only small portions should be mixed at a time, as the reagent (after mixing) does not keep, but undergoes gradual reduction.

The test for sugar in normal urine is made as follows:

First transfer 5-10 c.c. of the mixed reagent to a test tube, and boil the reagent in order to determine the extent, if any, to which the reagent is spontaneously reduced.

Transfer 1-2 g. picric acid to a small bottle; to it add some of the urine (10-50 c.c.) to be tested, insert a cork, shake for five minutes, and filter. By this treatment the disturbing creatinin and uric acid are removed. Now heat 5-10 c.c. of the copper solution to boiling in a fairly wide test tube (with a pebble to prevent bumping), add 1-2 c.c. of the filtered urine, and boil for 60-75 seconds. If the sugar present is relatively large in amount, the solution becomes turbid while still boiling, as in Benedict's test. If no such turbidity is observed, centrifuge at once, i.e., before cooling. The bottom of the centrifuge will be covered

with cuprous oxid. The crystals to be observed in the supernatant cooling liquid are potassium picrate.

Repeat this test with 1-2 c.c. urine which has not been treated with picric acid, and note how much more abundant the reduction is with ordinary creatinin-containing urine.

5. Bismuth Test.—Almen-Nylander's test for sugar depends on the ease with which alkaline solutions of bismuth are reduced. The reagent is made as follows:

To 4 g. of Rochelle salt in a small flask add 100 c.c. 10 per cent. caustic soda solution and 2 g. of bismuth subnitrate. Digest on a water bath for an hour. Let settle, and use the supernatant liquid.

This reagent is not used in the same way as the copper reagents. With the latter a little of the urine or sugar solution is added to 5-10 c.c. of the reagent. With the bismuth solution the proportions of reagent and urine are reversed.

To 5-10 c.c. of sugar solution add .5-1 c.c. of the bismuth solution, and heat (with a pebble) for 2-3 minutes. Set aside to cool spontaneously.*

The bismuth test is widely used, and is valuable for the detection of abnormal amounts of sugar in urine. Albumin interferes with the test, and when present must be removed. Certain drugs also interfere with the validity of the test.

6. Relative Sensitiveness and Significance of the Reduction Tests for Sugar.—With a .2 per cent. solution of dextrose determine the relative sensitiveness of the preceding qualitative tests for sugar.

Apply the same tests to concentrated normal urine (night urine). Record the results.

7. Phenylhydrazin Test (Osazone Test).—To 5-10 c.c. of .2 per cent. dextrose solution in a test tube add 5 c.c. of a phenylhydrazin solution (containing 5 per cent. phenylhydrazin hydrochlorid, 20 per cent. sodic acetate, and 10 per cent. acetic acid). Heat in a beaker of boiling water for half an hour. Let the test tube remain in the beaker until the water has cooled, and examine the glucose osazone crystals under the microscope.

Write the reaction involved in the formation of osazone.

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* See Hammarsten: "Almen Sugar Test."

8. Dextrose Reactions versus other Carbohydrates.—Apply one of the copper reducing tests, the bismuth test, and the phenylhydrazin test to .2 per cent. solutions of arabinose, levulose, cane sugar, maltose, and lactose.

Apply the copper reducing test also to .2 per cent. solutions of gum arabicum, commercial dextrin, and starch.

In the case of any of the above carbohydrate solutions which have yielded negative or doubtful results, digest 20 c.c. with 1 c.c. concentrated hydrochloric acid in boiling water for half an hour (or at room temperature over night), and repeat the test (or tests) made on the original solution. Tabulate and explain the results.

9. Selivanoff's Test for Ketose-Sugars.—Selivanoff's reagent contains .05 per cent. of resorcin and about 12 per cent. of hydrochloric acid.

To 5-10 c.c. of the reagent in a test tube add about 1 c.c. of .1 per cent. solution of levulose, boil for one minute, set aside to cool, and note the development of the color.

Repeat the test with dextrose and with cane sugar (and, if desirable, with other dilute sugar or carbohydrate solutions). Tabulate the results.

10. Test for Ketose Sugars (Levulose?) in Urine.—Collect urine for one hour (preferably just before the noon hour). Then take 50 g. of cane sugar and collect urine for another hour. Dilute the smaller volume of urine to that of the larger, or both to a convenient small volume. To 10 c.c. of each in a test tube add 5 c.c. of 10 per cent. lead acetate solution, shake, and filter. Apply Selivanoff's levulose test to about 2 c.c. of each filtrate. Record the results obtained.

Minute traces of levulose in human urine are probably not so infrequent an occurrence as is generally assumed to be the case. The color obtained with urine is, however, not exactly like that given by aqueous levulose solutions. If a distinct "levulose" output from the cane sugar is obtained, test both urines for reducing sugar, and note whether there appears to be an increase of sugar in the urine of the second period.

11. Orcin Test for Pentoses.—The pentose reagent is made by dissolving 1 g. of orcin in 500 c.c. 30 per cent. hydrochloric acid

(6:1) and adding 1 c.c. of 10 per cent. ferric chlorid solution.

Heat 5 c.c. of the orcin solution to boiling in a test tube. Remove from the flame, and add (immediately) 1 c.c. 1 per cent. solution of arabinose.

Repeat, adding 1 c.c. .2 per cent. arabinose solution.

Note the color (violet, blue, red, green) and the formation of a precipitate.

Repeat with 1 per cent. arabinose solution previously diluted (a) with 4 volumes of urine (b) with 4 volumes 1 per cent. glucose solution.

Repeat (a) with urine alone (b) with 1 per cent. glucose solution (c) with 1 per cent. cane sugar solution.

12. Fermentation Test for Sugar.—Certain sugars (which?) are decomposed by yeast into carbon dioxid and alcohol. The formation of CO_2 in fermentation has been extensively used both for qualitative studies and for quantitative determinations of sugar. Except among physicians who have not the facilities for making other tests, the fermentation method is now seldom used.

To some .5 per cent. sugar solution (dextrose, pentose, cane sugar, lactose) in a test tube add a small piece of yeast. Shake to make uniform mixtures, and with each fill a "fermentation tube." Substitute water for sugar solution in a control test, and set the tubes aside in a warm place over night. Record the results.

13. Benedict's Method for the Determination of Sugar.—Prepare 500 c.c. of Benedict's solution as follows: Dissolve 9 g. pure copper sulphate in a 500 c.c. volumetric flask with about 100 c.c. distilled water. Dissolve 50 g. anhydrous sodic carbonate, 100 g. sodium citrate, and 65 g. potassium sulphocyanate, in 250 c.c. distilled water. The copper sulphate must be weighed accurately on the analytical balance. Pour the copper solution, slowly, with stirring and without loss of a single drop, into the alkaline citrate solution. Then pour the mixed solution back into the measuring flask without loss, add 5 c.c. 5 per cent. potassium ferrocyanide solution, and with the rinsings make the total volume up to 500 c.c. Mix, transfer to a clean, dry bottle, label, and preserve. Twenty-five cubic centimeters of the solution corresponds to 50 mg. of dextrose, 52 mg. of levulose, 67 of lactose, or 74 of maltose.

The determination is carried out as follows:

Measure 25 c.c. of Benedict's solution into a porcelain dish, add 5-10 g. of solid sodic carbonate, heat to boiling, and while boiling run in the sugar solution (or urine) fairly rapidly until a white precipitate begins to form. Then add the solution more slowly (with slower boiling) until the last trace of the blue color disappears. The addition of the sugar solution should be done at such a speed that the boiling solution is kept nearly constant in volume during the operation. The original sugar solution (or urine), if concentrated, should be diluted so that not less than 10 c.c. will be required to give the amount of sugar which the 25 c.c. of reagent is capable of oxidizing.

Five divided by the volume of sugar solution taken gives the per cent. of sugar. Check the value of the reagent by determining the sugar in .5 per cent. dextrose solution.

14. Polariscope Method for the Determination of Sugar.—The specific rotation of a substance is the angle through which the plane of polarized light is turned by 1 dm. of a solution containing 1 g. of the optically active substance per c.c.

A definite temperature and light of a definite wave length (sodium light) must be used in determining specific rotations.

The angle of rotation is determined by means of some form of "polariscope" (polarimeter, saccharimeter, etc.); and the specific rotation is calculated according to the following formula:

$$\text{Specific Rotation} = \frac{\text{Observed Rotation} \times 100}{\text{Percentage} \times \text{Length of Observation Tube (dm.)}}$$

If the specific rotation is known, as in the case of the common sugars, the per cent. of sugar is obtained by the following transposition of the above formula:

$$\text{Percentage} = \frac{\text{Observed Rotation} \times 100}{\text{Specific Rotation} \times \text{Length of Tube (dm.)}}$$

Following are the specific rotations (yellow light) of some common sugars:

Glucose, 52.8; Fructose, —90; Cane Sugar, 66.5; "Invert Sugar," —20; Lactose, 52.4; Maltose, 137.

The determination of sugar by means of the polariscope is as follows:

Rinse the polariscope tube (length usually 1 or 2 dm.) with the sugar solution, and fill almost to overflowing. Place the glass plate over the open end in such a way that the tube does not contain any air bubble, and screw on the cap. Place the tube in the groove of the polariscope. Light the lamp, and move the eyepiece back and forth until the lines which divide the field are seen sharp, without effort. Then turn the screw until the several divisions of the field are equally illuminated, and take a reading by means of the vernier. The circle upon the disk of the apparatus is divided into quarter degrees; 24 divisions of the vernier correspond in length to .25°. Consequently every division of the vernier corresponds to .01°. Ascertain whether the disk, starting from its middle point, has been moved to the right or to the left of the zero point of the vernier. Read off the number of whole degrees and hundredths. Take several readings by moving the lever and coming back again to the point where the different parts of the field are equal. Correct for the zero point of the instrument by taking readings with the tube filled with water. This value is added to, or subtracted from, the reading found with the sugar solution.

If a saccharimeter is used instead of the general circular polariscope, the reading on the scale is converted into angular degrees of rotation by multiplying by the factor .345. The percentage of sugar in the solution is then calculated by using the formula given above.

Prepare 100 c.c. dextrose solution containing about 5 per cent. of sugar. The amount of sugar taken should be weighed accurately. To overcome the "muta rotation" of the dextrose solution either heat the solution to 100° and cool, or allow to stand over night (or add first one drop of ammonia and then one drop of acid).

Determine the sugar content of the solution by the polariscope, and also (after appropriate dilution) by titration with Benedict's copper solution.

Determine by titration, and also by the optical rotation, the dextrose content of three unknown "sugar urines."

Before the sugar in urine can be determined by the polariscope it is often necessary to "clear" it by the addition of a pinch of basic lead acetate and filtration.

15. Cane Sugar.—Prepare 100 c.c. of a 5 per cent. cane sugar solution by the help of the balance. Determine the concentration of the solution with the polariscope.

Transform a part of the solution into "invert sugar," and determine the sugar content by titration with Benedict's solution.

The inversion is carried on as follows:

Transfer to a 100 c.c. volumetric flask 10 c.c. of the cane sugar solution, and add 5 c.c. of water and 1 c.c. concentrated hydrochloric acid. Heat water in a large beaker or porcelain dish to 70° C. When the water bath has reached 70° C., immerse the flask in the water, rotate it continuously for ten minutes, and at once dilute to the 100 c.c. mark with cold water. Mix, and titrate the sugar content as in the case of dextrose, giving 25 c.c. of the copper solution a value of 51 instead of 50 mg. of sugar (why?).

16. Problem.—On the basis of the tests and determinations made with dextrose, levulose, pentose, and cane sugar, work out a scheme for identifying (a) dextrose and levulose (b) dextrose and pentose (c) dextrose and cane sugar—when any one of these pairs is present. Apply to the unknowns furnished.

17. Preparation of Maltose.—Mix 10 g. of starch with 30 c.c. of cold water until a smooth paste is obtained. Pour this slowly, and with stirring, into 250 c.c. of boiling water in a large beaker, continue the boiling 1-2 minutes, let cool to 75° C., stir in 1 teaspoonful of malt, and keep at this temperature until the mixture becomes thin and watery. Heat again to boiling with stirring, cool to 75°, add another teaspoonful of ferment, and keep at this temperature until a sample no longer gives any color with iodin solution (about two hours). Boil, filter (taste), and determine the sugar quantitatively in a sample of the filtrate (a) as it stands, (b) after heating on the water bath with hydrochloric acid for about two hours. Explain.

18. Preparation of Milk Sugar.—To 300 c.c. of skimmed milk diluted with 800 c.c. of water, add cautiously 2 per cent. acetic acid to precipitate the casein. When enough has been added the liquid is nearly clear. Filter. Boil the filtrate, and filter off the coagulated albumin. Evaporate the filtrate on the water-bath to a thin syrup, and allow it to stand until the sugar has crystallized out.

19. Starch.—Rub 2 g. of starch to a thin paste with a little water, and pour the mixture slowly, with constant stirring, into 300 c.c. of boiling water.

To 5 c.c. of the starch paste and 10 c.c. water in a test tube add a drop of iodin solution. Note the result. Heat the solution gradually. What change takes place? Cool and observe the result. Then boil and again allow to cool.

Add a few drops of a solution of tannic acid to a few cubic centimeters of the dilute starch solution. Warm.

Test a portion of the starch solution for reduction.

To 25 c.c. of saliva in a small flask add 25 c.c. of the starch paste. Mix, and test a small portion for sugar. Digest the remainder at 40° C., and test for sugar and for starch at the end of 15 minutes, 30 minutes, and two hours.

Mix a little uncooked starch with saliva, expose to a temperature of 40° C. for fifteen minutes, and test for sugar.

20. Dextrin.—Dissolve a little commercial dextrin * in water, and make the reduction test and the test for starch.

Saturate 5 c.c. of the dextrin solution with solid ammonium sulphate. Filter. Test the filtrate with iodin. Remove precipitate from filter, dissolve in a little hot water, cool, and test with iodin.

Boil 5 c.c. of the solution with an equal volume of dilute (10 per cent.) hydrochloric acid for 5 minutes. Test for sugar. Save the remainder of the dextrin solution for comparison with glycogen.

Test Graham cracker or toast for dextrin by extracting with cold water, filtering, and applying the iodin test.

21. Glycogen.—Cut four fresh (Why fresh?) oysters into small pieces, and throw into four times their weight of boiling water slightly acidulated with acetic acid. After boiling for a short time, remove the pieces, grind in a mortar with some sand, return to the water, and continue the boiling for several minutes. Filter while hot. The opalescent solution thus obtained is an aqueous solution of glycogen and other substances.

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* Commercial dextrin sometimes contains unaltered starch, and usually contains reducing sugar; in the former case iodin may give a blue or violet (mixture of red and blue) color; in the latter a reduction takes place in Fehling's test. Dextrin gives no reaction with Fehling's solution.

With the solution of glycogen thus obtained, make the following tests:

Add iodin solution drop by drop to a portion of the glycogen solution. The liquid will assume a dark red color. This color disappears, with the exception of the color due to the iodin, upon gentle heating, and reappears upon cooling. (Compare with dextrin.)

Test the glycogen solution with Benedict's solution and note the result.

Add some saliva to a portion of the glycogen solution, and put in the warm room until the next day. Remove and divide into two portions. Test one with iodin solution for glycogen, the other for sugar. Report the result.

PART V

PROTEINS

A fundamentally important general consideration to be noted in the study of protein materials is that proteins are colloids, and that colloidal solutions differ materially in their physical and chemical reactions from the corresponding reactions of ordinary true solutions (of crystalloids).*

1. Dialysis of Colloidal and Molecular Solutions.—In a parchment dialyzing tube place 15 c.c. of blood serum (protein) and 1 c.c. of strong salt solution, Na_2SO_4. The protein is present in colloidal form, the salts in molecular (ionic) solution. Suspend the tube in a 500 c.c. beaker of distilled water, and let the whole stand over night. Test a portion of the dialysate for protein by boiling in a test tube with one drop of dilute acetic acid. Has protein passed through the membrane? Test the dialysate for chlorids and sulphates. Do salts dialyze through parchment membranes?

2. Suspension Colloids; Suspensoids.—Preparation.—Prepare a colloidal solution of gum mastic as follows: Drop from a buret 1 c.c. of saturated alcoholic solution of gum mastic, slowly and with stirring, into 100 c.c. of distilled water. Filter.

Action of Electrolytes.—To 10 c.c. portions of the above filtrate add

1. 5 c.c. tenth normal HCl,
2. 5 c.c. tenth normal NaOH,
3. 5 c.c. normal NaCl,
4. 5 c.c. urea solution,
5. 5 c.c. concentrated cane sugar solution.

Let stand, note what occurs, and explain.

Effect of Heat.—Boil a portion of the solution in a test tube.

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* Fats and the more complex carbohydrates behave also as colloids; so indeed do all substances which are insoluble in water, when suspended in water in a state of sufficiently fine division. Colloidal solutions of metallic platinum (one of the least soluble of metals) are frequently employed in the study of certain ferment reactions. Proteins occur in nature almost wholly in the form of colloidal solutions.

Evaporate 10 c.c. of solution to dryness on the water bath, and try to redissolve it.

3. Emulsion Colloids; Emulsoids.—PREPARATION.—To 5 g. gelatin in a beaker add 60 c.c. of water, and heat gently with constant stirring until the gelatin is all dissolved.

ACTION OF ELECTROLYTES.—To 10 c.c. portions of the gelatin solution add,

1. A few drops 10 per cent. NaCl,
2. A few drops concentrated HCl.

Evaporate 10 c.c. to dryness on the water bath, and try to redissolve.

REVERSIBILITY OF COAGULATION BY ELECTROLYTES.—To 25 c.c. of a protein solution add solid ammonium sulphate with shaking until the solution is saturated, and note what happens. Filter and press the precipitate as dry as possible between layers of filter paper. Try to redissolve the precipitate in water.

REVERSIBILITY OF COAGULATION BY HEAT.—To another 25 c.c. portion of the same solution add 2-3 drops dilute acetic acid. Heat to boiling. Filter, press the precipitate dry as before, and try to redissolve in water. Tabulate the differences between emulsoids and suspensoids.

4. "Hydrophile" Colloids.*—To some gelatin in a test tube add just sufficient water to cover it. Let stand and note changes.

5. Reversibility of "Sol" and "Gel" States—Gelatinization as a Special Case of Coagulation.—Warm the above mixture until solution is complete. Cool under the cold water tap. What occurs? Repeat the heating and cooling a number of times.

6. Positively and Negatively Charged Colloids.—Place a few c.c. of colloidal $Fe(OH)_3$ solution in one small beaker, and a few c.c. of colloidal As_2S_3 solution in another. Hang a long, narrow strip of filter paper over each beaker so that the lower end touches the liquid. Let stand over night. Which colloid rises to the greater height? Explain.

7. Coagulation by Oppositively Charged Colloids.—To 10 c.c. colloidal arsenic solution add 10 c.c. colloidal $Fe(OH)_3$. Let stand and note what occurs.

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* "Hydrophile" colloids are one class of emulsoids—not all emulsoids are "hydrophilous."

8. Rate of Diffusion of Molecular and Colloidal Solutions.—Melt some 4 per cent. gelatin or 2 per cent. agar jelly in a beaker by standing in hot water. Fill 5 small test tubes 1/3 full and allow to solidify. To the jelly tubes, 1-5 respectively, add an equal volume of

1. Colloidal As_2S_3,
2. Colloidal $Fe(OH)_3$,
3. Picric acid,
4. Copper sulphate,
5. Congo red.

Allow to stand 2 days. Compare the speed of diffusion, i.e., the distance which the various substances have penetrated the jelly. What is the relation between the size of particles and rate of diffusion?

1. **Test for Nitrogen, Sulphur, and Phosphorus in Protein.**—Put a little dry protein * into a dry, cheap test tube. Add a piece of metallic sodium the size of a pea, and heat strongly for a few minutes. Cool. Carefully and without handling the material, break into a dry evaporating dish. Cover the substance with a wet filter paper. After five minutes cautiously add 25 c.c. water. Stir well. Filter into a test tube.

(a) To 5 c.c. add a few drops of ferrous sulphate and a drop of ferric chlorid solution. Warm and acidify with concentrated hydrochloric acid, noting the result.

(b) Acidify 5 c.c. with nitric acid and add a few drops of ammonium molybdate solution. Let stand over night and look for a yellow crystalline precipitate.

(c) To another 5 c.c. add a few drops concentrated sulphuric acid, and suspend over the mouth of the test tube a piece of filter paper previously moistened with lead acetate solution.

Discuss the action of the sodium, and the chemistry of the tests (a), (b), (c).

2. **Simple Test for "Amid" Nitrogen and for Sulphur.**—Put a little protein in a test tube with a few cubic centimeters of strong sodium hydrate solution and three drops lead acetate solution. Heat to boiling, and suspend a piece of litmus paper over the mouth of the test tube. Explain.

3. **Test for Phosphorus.**—Boil some casein in the hood with 10 c.c. strong nitric acid in an evaporating dish. Evaporate nearly to dryness, add 25 c.c. water, and test for phosphates.

4. **Albumins.**—Preparation of an albumin solution. Considering "egg white" to contain 12 per cent. of albumin, prepare a 2 per cent. solution by suitable dilution with distilled water. Shake thoroughly, and filter through a plug of cotton.

5. **Coagulation by Heating.**—Heat a little of the albumin solution in a test tube. Compare the coagulation so obtained with

* A mixture of casein and dry egg albumin.

that obtained when the solution is diluted (a) 20 times with distilled water, (b) 20 times with .1 per cent. salt solution, (c) 20 times with .01 per cent. acetic acid, (d) 20 times with equal volumes of .1 per cent. salt solution and .01 per cent. acetic acid solution.

6. **Coagulation Temperature.**—Ascertain the temperature of coagulation of albumin as follows: Faintly acidify a portion of the solution with a few drops of .5 per cent. acetic acid. Filter if necessary. Place a portion of the solution in a test tube, insert a thermometer by means of a cork, and suspend the tube in a large beaker of water. Heat the beaker slowly with a small flame, and observe the point at which the albumin solution becomes cloudy.

7. **Sulphosalicylic Acid Test.**—To some albumin solution in a test tube add a few drops of sulphosalicylic acid solution (25 per cent.). Determine the delicacy of the test.

8. **Nitric Acid Test.**—Put 5 c.c. of the solution in a test tube, and introduce 5 c.c. of concentrated nitric acid very carefully with a pipet to the bottom, forming an under layer. Determine the lowest protein concentration at which the test is unmistakable. Allow 10 minutes if the reaction is slow in appearing.

9. **Picric Acid Test.**—Add to a portion of the albumin solution a few drops of a solution of picric acid (1 per cent.) and citric acid (2 per cent.)—Esbach's reagent. Determine the lowest protein concentration at which this test is unmistakable.

10. **Action of Ammonium Sulphate.**—Add some solid ammonium sulphate to 10 c.c. of the albumin solution in a test tube, shaking frequently until the solution is thoroughly saturated. Allow to stand for a while, occasionally shaking, filter, and test the filtrate for albumin by the heat test. Test the solubility in water of the precipitate on the filter paper.

11. **Action of Magnesium Sulphate.**—Perform a similar experiment, using solid magnesium sulphate instead of ammonium sulphate. To a portion of the filtrate add one or two drops acetic acid.

12. **Biuret Test.**—To a portion of the albumin solution add

a little sodium hydrate, then, drop by drop, very dilute copper sulphate. The solution becomes violet. Study the delicacy of the reaction. After adding to the albumin solution some solid ammonium sulphate, repeat the test with (a) the same amount of alkali (b) a large amount of 40 per cent. alkali.

13. **Millon's Test.**—To a portion of the albumin solution add a few drops of Millon's reagent. A precipitate forms, which, on heating, becomes brick red. Repeat, using a dilute solution of phenol instead of albumin. On what group in the protein molecule does this test depend? Add sodium chlorid and repeat the test. Explain.

14. **Xanthoproteic Test.**—To a few c.c. of the solution add one-third of its volume of concentrated nitric acid; a white precipitate may or may not be produced (according to the concentration and the nature of the protein). Boil. The precipitate or liquid turns yellow. Allow the solution to cool, and add an excess of ammonia. Explain.

15. **The Glyoxylic Acid Reaction (Hopkins and Cole).**—Treat 2 or 3 c.c. of the solution with the same volume of "reduced oxalic acid." Mix and add an equal volume of concentrated sulphuric acid, pouring down the side of the tube. A purple ring forms at the junction of the fluids. Mix the fluids by shaking the tube gently from side to side. The purple color spreads through the whole fluid. Repeat in the presence of nitrates, chlorates, nitrites, excess of chlorids, and carbohydrates, respectively.

"Reduced oxalic acid" is prepared by Benedict's method as follows: To 10 g. powdered magnesium in a flask add a little water, and then add slowly, with shaking and cooling, 250 c.c. of cold saturated oxalic acid solution. Filter, acidify the filtrate with acetic acid, and dilute to one liter.

16. **Acetic Acid and Potassic Ferrocyanid.** —Acidify some albumin solution in a test tube with acetic acid, and add a few drops of potassic ferrocyanid solution. A white flocculent precipitate is formed. Determine the delicacy of this precipitation.

17. **Alcohol.**—Add an excess of alcohol (one or two volumes) to some albumin solution. If the precipitate is small, add a little dilute sodium chlorid solution.

18. Tannic Acid.—Make some protein solution slightly acid with .1 per cent. of acetic acid, and add a few drops of tannic acid solution.

19. Phosphotungstic Acid.—Make a protein solution acid with dilute hydrochloric acid, and add a few drops of the reagent.

Globulins.—The tests are made upon blood serum.

20. Action of Carbon Dioxid.—Dilute 5 c.c. of clear serum with 45 c.c. of ice-cold water. Place the mixture in a cylinder or large test tube, and pass through it a stream of carbon dioxid. What is the effect of too much carbon dioxid?

21. Precipitation by Dialysis.—Pour 20 c.c. of serum into a parchment dialyzing tube previously soaked in distilled water. Suspend the tube, with its contents, in a large volume of water. Explain the precipitation.

Pour serum drop by drop into a large volume of distilled water (in a beaker). What takes place? Explain.

22. Precipitation by Magnesium Sulphate.—Saturate about 5 c.c. of the serum with magnesium sulphate. A heavy precipitate will be formed. Compare this with the action of the same salt on the egg-albumin solution.

23. Precipitation with Ammonium Sulphate.—To 30 c.c. of serum add an equal volume of a saturated solution of ammonium sulphate, thus obtaining a half-saturated solution. Filter off the precipitate, wash two or three times with a half-saturated ammonium sulphate solution, and dissolve in about 60 c.c. of water. This yields a clear solution of paraglobulin. Apply 5 protein tests to this solution.

24. Keratin.—Show that keratin (hair or horn) is a protein.

25. Gelatin.—Make dilute gelatin solution, and with it make 6 tests for protein (including Millon's). Test for sulphur.

26. Phosphoproteins.—Test the solubility of casein in water, dilute acid, dilute alkali, and dilute salt solution.

Make six protein tests on a solution of casein. How would you test for albumin and casein when both are present? Apply to milk and to unknown furnished.

27. Peptones (Proteoses).—For the following experiments use the peptic digestion mixture obtained with the pig stomach (p.

18. **Tannic Acid.**—Make some protein solution slightly acid with [illegible] per cent. of acetic acid, and add a few drops of tannic acid solution.

19. **Phosphotungstic Acid.**—Make a protein solution acid with [illegible] hydrochloric acid, and add a few drops of the reagent.

Globulins.—The tests are made upon blood serum.

20. **Action of Carbon Dioxid.**—Dilute 5 c.c. of clear serum with [illegible] c.c. of ice cold water. Place the mixture in a cylinder or large [illegible] tube, and pass through it a stream of carbon dioxid. What is the effect of too much carbon dioxid?

21. **Precipitation by Dialysis.**—Pour 20 c.c. of serum into a parchment dialyzing tube previously soaked in distilled water. Suspend the tube, with its contents, in a large volume of water. Explain the precipitation.

Pour serum drop by drop into a large volume of distilled water (in a beaker). What takes place? Explain.

22. **Precipitation by Magnesium Sulphate.**—Saturate about 5 c.c. of the serum with magnesium sulphate. A heavy precipitate will be formed. Compare this with the action of the same salt on the [illegible] solution.

23. **Precipitation with Ammonium Sulphate.**—To 30 c.c. of serum add an equal volume of a saturated solution of ammonium sulphate, thus obtaining a half saturated solution. Filter off the precipitate, wash two or three times with a half-saturated ammonium sulphate solution and dissolve in about 60 c.c. of water. Dissolve [illegible] of [illegible]. Apply a protein test [illegible].

24. **Serafin.**—Shown that serafin (hair or horn) is a protein.

25. **Gelatin.**—Make [illegible] solution, and with it make [illegible] (Millon's). Test for sulphur.

26. **Phosphoproteins.**—Test the solubility of casein in water, dilute acid, dilute alkali, and dilute salt solution.

Make six protein tests on a solution of casein. How would you test for albumin and casein when both are present? Apply to milk and to unknown furnished.

27. **Peptones (Proteoses).**—For the following experiments use the peptic digestion mixture obtained with the pig stomach (p.

—). Filter, carefully neutralize, heat to boiling (why?), and again filter. Use the filtrate.

To a small portion add 2 or 3 drops of dilute acetic acid and a few c.c. of saturated sodium chlorid solution. Study the effect of heating and cooling on this precipitate.

Apply protein tests described under "albumin," and record the results obtained.

Dialyze about 10 c.c. of the peptone solution against about 100 c.c. of distilled water in a beaker. After 24 hours test the outside water for peptones. Explain.

28. Amino-acids, Tyrosin and Leucin.—For this experiment use the pancreatic digestion mixture prepared for the study of ferment reactions (p. 27).

With a pipet take out 10 c.c. of clear supernatant liquid. Filter this portion if necessary; dilute it with 2 volumes of water, and by means of Mett's tubes determine whether the proteolytic ferment has been destroyed or is still active.

Pour the rest of the digestion mixture without filtering into a good-sized beaker or flask. With continuous stirring or gentle shaking to prevent burning and bumping, heat the digestion mixture until it begins to boil. Some care is needed in this operation because of the presence of alcohol. When the mixture is boiling remove the flame. Note approximately the volume of the mixture, and measure into a test tube some "Merck's dialyzed iron," 8-10 c.c. for each 100 c.c. of digestion mixture. Dump the colloidal iron into the digestion mixture, and shake or stir vigorously. Filter.

To the filtrate add a few drops of ammonia and 5-10 g. of bone-black. Boil for a few minutes and again filter. A clear, faintly-colored solution should be obtained.

Pour this last filtrate into an evaporating dish, acidify with a little acetic acid, and boil down to about one-sixth of the original volume.

Transfer the concentrated liquid to a flask or beaker, and set aside in a cool place for a day or two. Tyrosin and leucin crystallize out, the former first and in much greater abundance. Examine the sediment under the microscope.

The isolation of other amino-acids from the mother liquor is much more difficult.

29. Preparation of Cystin (from Wool).—Heat 50 g. of wool in a 500 c.c. flask with 100 c.c. concentrated hydrochloric acid on a waterbath until dissolved. A 3-foot glass tube should be inserted to prevent the loss of too much acid liquid. When dissolved boil very gently over a *small* flame for 3-4 hours. Add solid sodic acetate (100-130 g.) until no free mineral acid can be detected in the solution by means of congo red paper. Allow the mixture to stand for 3-5 days. The longer the mixture is allowed to stand, up to 3 weeks, the more cystin is obtained. Filter on a Buchner funnel and wash with cold water. Then dissolve the precipitate in water (150 c.c.) plus 5-10 c.c. concentrated hydrochloric acid, add about 20 g. purified bone-black, and boil 5-10 minutes.

To prepare pure bone-black, let the impure sample stand in an excess of dilute hydrochloric acid over night, filter, and wash with cold water until the filtrate is neutral.

Filter again with suction, heat the filtrate to boiling, and neutralize the hot hydrochloric acid by adding very slowly hot concentrated sodic acetate solution (avoid an excess, test with congo red paper). The precipitate formed consists of cystin, and should be very white and pure. If it is dark colored, re-dissolve in water and a little hydrochloric acid, and repeat the bone-black treatment.

Keep the mixture boiling, and add very slowly the hot sodic acetate solution until the crystallization begins; keep hot, and after a few minutes add cautiously a little more acetate. Well-formed, large, and characteristic crystals should be obtained.

Examine the crystals under the microscope.

Test for sulphur. Test also for tyrosin. (Ordinarily the crystals are quite free from tyrosin.)

Tyrosin can be prepared from the original mother liquor by decolorizing with bone-black and letting it stand in a cold place.

PART VI

URINE ANALYSIS AND METABOLISM

Each student is expected to keep on hand accurately collected and measured twenty-four hour specimens of his own urine. The urines should be preserved by the addition of 5 c.c. of chloroform to each bottle. All determinations should be made in duplicates.

1. Aëration Method for the Determination of Ammonia.—Measure 25 c.c. of the ammonium sulphate solution previously used for nitrogen determinations (p. 17) into a tall aërometer cylinder. The cylinder is fitted with a two-hole rubber stopper and glass connections so arranged that compressed (outside) air (or laboratory air previously freed from ammonia) is passed to the bottom of the cylinder and out through a calcium chlorid tube filled with dry cotton, and then through a special absorption tube, into a receiver containing water and a known amount of acid. The cotton serves the purpose of holding back traces of solid sodic carbonate, formed by evaporation on the sides of the cylinder.

Add to the ammonium sulphate solution about 10 g. sodium chlorid, about 2 g. sodic carbonate, and a few drops of kerosene. Do not add any water; the greater the volume the longer it takes to drive off all the ammonia. Pass a very strong air current through the mixture for one and one-half hours, and collect the ammonia, which the air carries off, in a receiver containing 25 c.c. .1 N acid and about 200 c.c. water. Titrate, and compare the result with the figures obtained by distillation (p. 21). If the results are too low, the air current has been too slow, or the aëration process has not been continued long enough to drive off all the ammonia.

In a similar manner determine the ammonia in urine (25 c.c.) and calculate the 24-hour amount. When working with urine it is desirable, though not absolutely necessary, to substitute 10 g. of

potassium oxalate for the 10 g. of sodium chlorid. Salts hasten the removal of the ammonia, and oxalate incidentally prevents the (possible) formation of insoluble ammonium magnesium phosphate.

Save the remainder of the ammonium sulphate solution for sulphate determinations.

2. Colorimetric Method for the Determination of Ammonia.—With an Ostwald pipet measure 1 or 2 c.c. of the ammonium sulphate solution into a large Jena test tube (200 x 25 mm.). Choose the amount which contains nearer 1 mg. of nitrogen. Fit the test tube with a two-hole rubber stopper carrying an inlet tube, reaching to the bottom, and an outlet tube. Connect the former with the compressed air jet, and the latter with an absorption tube having small holes drilled through the wall at the end. Insert the absorption tube into a 100 c.c. measuring flask containing 20-30 c.c. of distilled water and 2 c.c. .1 N HCl. Add 2 drops kerosene and a few drops of a solution containing potassium oxalate and potassium carbonate (15 per cent. of each), quickly put the stopper firmly in the tube, and start the air current, gradually increasing its speed for about two minutes. In ten minutes all the ammonia should have been driven over into the receiving flask.

Remove the absorption tube, rinsing it with water, and dilute the contents in the flask to about 60 c.c.

Pipet 1 c.c. of standard ammonium sulphate, using the Ostwald pipet, into another 100 c.c. measuring flask, and dilute with water to 60 c.c.

Nesslerize both solutions simultaneously in the following manner: Provide two small beakers (100 c.c.), and place 20-25 c.c. of distilled water in each. Add to each 5 c.c. of Nessler's reagent. Mix the reagent with the water, and add it immediately to the ammonia solutions (about one-third of the diluted Nessler reagent at a time). Shake after each addition.

Fill both flasks up to the mark with distilled water and mix. The resulting deep red colloidal solutions must be absolutely crystal clear. If the least turbidity or opalescence is perceptible in either flask, that flask must be discarded. When properly made the Nesslerized solutions remain clear and unchanging for several hours.

The colorimetric value of the unknown solution is next deter-

mined by means of a Duboscq colorimeter. In this process the standard solution, set at 20 mm. in the colorimeter cups, must first be read against itself. The readings obtained in such comparisons should come within .2 mm. of the theoretical figure (20 mm.). When the instrument (and the eye) are thus adjusted, rinse out the contents of one of the colorimeter cups with the unknown solution, and repeat the color comparison, taking from 3 to 6 separate readings. The ammonia content is inversely proportional to the depth of the color. Twenty divided by the reading gives the ammonia nitrogen (in mg.) in the volume of ammonium sulphate solution taken. Calculate the result and compare with the figures previously obtained (a) by distillation (p. 21), (b) by the macro-aëration process (p. 79).

Repeat the ammonia determination described above, with urine. The volume of urine taken should give .75-1.25 mg. of ammonia nitrogen (1-5 c.c.). Calculate the 24-hour quantity of the ammonia thus obtained, and compare with the figure obtained by the preceding method.

3. Clinical Method for the Determination of Ammonia.—The special reagents required in this determination are (a) saturated potassium oxalate solution and (b) formalin; both of which must be neutral to phenolphthalein. To each reagent add a little of the indicator and .1 N alkali to a faint pink coloration.

To 25 c.c. of urine add about 5 c.c. of the neutralized oxalate solution and 2-3 drops phenolphthalein solution. Titrate the acidity of the urine to a *faint* but unmistakable end point. Then add about 5 c.c. of the neutralized formalin and again titrate to the same degree of coloration as in the preceding titration. Each c.c. of the .1 N alkali used in this titration corresponds to 1 c.c. .1 N ammonia.

The formaldehyd combines with the ammonia giving neutral hexamethylene tetramin, thus setting free acid equivalent to the amount of ammonia present.

4. Total Nitrogen. Colorimetric Method.—Special equipment called for: (a) Duboscq colorimeter, (b) standardized 1 c.c. "Ostwald pipets," with extra long stems, (c) a solution of specially purified ammonium sulphate, of such strength that 1 c.c. contains 1 mg. of nitrogen (4.7062 g. salt per liter), (d) modified Nessler-Winkler reagent (see p. 161).

Consult the instructor before beginning this determination.

Dilute the urea solution previously used for N determinations (p. 21) so that 1 c.c. contains approximately 1 mg. of nitrogen.

Pipet 1 c.c. of the diluted urea solution, using the Ostwald pipet, into a large Jena test tube. Add 1 c.c. concentrated H_2SO_4, 1 g. K_2SO_4, 1 drop $CuSO_4$ solution (10 per cent.), and a small pebble or glass bead.

Boil over a micro-burner for 10 minutes. Allow to cool until the digestion mixture just begins to become viscous (it must not solidify); then add about 6 c.c. of water, at first a few drops at a time, then more rapidly if the digestion mixture begins to solidify.

Draw up 3 c.c. of saturated sodic hydrate solution into the long glass tube carried in the two-hole rubber stopper, and retain by means of a piece of rubber tubing and a pinchcock. Stopper the test tube with this apparatus, and connect the outlet tube to an absorption tube having several small holes drilled through the wall at the end. The absorption tube is placed in a 100 c.c. measuring flask, containing 20-30 c.c. of distilled water and 2-5 c.c. tenth normal HCl.

Release the pinchcock, thus allowing the alkali to flow into the test tube. Connect with the compressed air, and turn on carefully so that one or two bubbles mix the solutions. Run the air slowly for two minutes, then rapidly for ten minutes, just as in the ammonia determination. At the end of this time all of the ammonia should have gone over.

Remove the delivery tube, rinsing it with water, and dilute the contents in the flask to about 60 c.c. Complete the determination as in the case of ammonia.

Calculate the results, and compare with the figures obtained by Kjeldahl's process (p. 22).

Repeat the determination with diluted urine (1-5 or 1-10). Compare with a Kjeldahl determination (5 c.c. urine) upon this same urine undiluted.

Save the original urea solution for direct urea determinations.

5. Reactions of Urea.—Put a crystal of urea on a glass slide or a watch-glass and cover it with a drop of water. With a glass rod, put a drop of nitric acid next to this. Let the two drops run together, and notice the precipitation of urea nitrate at the

junction. Examine under the microscope, and sketch the crystals.

Put a few crystals of urea into a dry test tube, and heat till they melt. With moist litmus paper test the reaction of the fumes given off. Explain.

Cool the test tube. To the residue, consisting of biuret and cyanuric acid, add a little water, filter, and with the filtrate make the biuret test.

Dissolve a few crystals of urea in 5 c.c. water in a test tube. Test its reaction with litmus paper. Heat the solution to boiling and test the steam with moist litmus paper. Cool the liquid and test with litmus paper. Explain.

To a solution of urea in a test tube add an equal volume of sodium hypobromite solution. Make this by mixing and cooling equal volumes of bromin solution and 40 per cent. sodic hydrate solution.

The reaction with sodium hypobromite has been used for the quantitative determination of urea, but as ordinarily used for this purpose the method has very little value.

6. Colorimetric Method for the Determination of Urea (*J. Biol. Chem., II, 507*).—Measure 1 c.c. of the urea solution used for nitrogen determinations (diluted 1-5 or 1-10) into a large dry Jena test tube. Add 1 c.c. 50 per cent. acetic acid, 7 g. dried potassium acetate, a pebble, and a temperature indicator (described below). Insert through a rubber stopper, as condenser, a special long calcium chlorid tube. Then heat with a microburner until a clear solution is obtained and the indicator melts. This should happen in two or three minutes. Add water first through the condenser, and then directly into the tube after taking out the rubber stopper. The sides of the tube should be rinsed carefully without using more than 5 or 6 c.c. of water, without letting the contents in the test tube solidify, and without losing ammonia (which will occur if the stopper is taken out too soon).

Add to the contents of the tube about 2 c.c. 30-40 per cent. caustic soda and drive out the ammonia, as in the total nitrogen determination with the air current. If the total volume in the test tube does not exceed 10 c.c. the ammonia will go over in 10 minutes. Nesslerize and compare with standard ammonium sulphate as before.

The temperature indicator is HgICl, a mixed halid prepared by heating together equivalent quantities of mercuric chlorid and mercuric iodid to 150-160° C., for 3-4 hours. This compound melts at 153° C. It cannot be purified, hence the salts taken should be weighed out with care. .1-.2 g. of the compound is sealed up in small glass bulbs.

7. Marshall's Urease Method for the Determination of Urea.—PREPARATION OF ENZYME.—Grind 25 g. of soy beans in a coffee mill. Extract the meal with 125 c.c. water for one hour, shaking frequently. Add to the mixture about 25 c.c. approximately decinormal hydrochloric acid and let stand for 5 minutes. Filter with suction on a Buchner funnel. To the filtrate add 5 c.c. of a solution made by dissolving 70 g. disodic phosphate and 27 g. monopotassium phosphate in 100 c.c. of water. Shake until the phosphates are dissolved and dilute to a volume of 200 c.c. In the cold this enzyme solution keeps its strength for several weeks.

THE DETERMINATION.—To 1 c.c. of urea solution or urine (so diluted as to contain about 1 mg. urea-nitrogen per c.c.) in a large test tube add 1-2 c.c. of the enzyme extract (a large excess is undesirable). Add 2 drops of kerosene, and immediately stopper with the aëration apparatus used for ammonia or other nitrogen determinations. Let stand for 15 minutes. Now pass the current through the mixture for about half a minute to drive off any free ammonia which may be present, collecting it as usual in a 100 c.c. volumetric flask containing 25 c.c. water and 2 c.c. decinormal hydrochloric acid. Stop the air current, open the tube, and add about 1 c.c. of potassium carbonate oxalate solution (as in the preceding method).

Close the tube promptly, start the air current again, gradually increasing the speed and running it very fast for 10-15 minutes.

The subsequent Nesslerization and color comparison, with 1 mg. of ammonia-nitrogen as the standard, are made in the usual manner.

Repeat the urea determination on two twenty-four hour urines. Calculate the urea nitrogen and urea in each sample, making due allowance for the ammonia of the urine.

8. Uric Acid Preparation of Uric Acid from Urine.—To 500 c.c. of urine add 25 g. of ammonium sulphate, stir until the sulphate is dissolved, and add about 10 c.c. of ammonia. Let stand over night, filter, and wash two or three times with water con-

taining a little ammonia. Transfer the precipitate to a small beaker, add a few drops of hydrochloric acid, and let stand till the following day. Examine the crystals under the microscope.

9. Murexid Test for Uric Acid.—Place a few uric acid crystals on a porcelain crucible cover. Add three drops of strong nitric acid. Heat cautiously, blowing on the liquid to complete dryness. A red color should appear. Let cool and add a few drops of dilute ammonia. Repeat with caffein. Explain.

10. Phosphotungstic Acid Test for Uric Acid.—Dissolve a few crystals of uric acid in 2 c.c. very dilute sodic hydrate solution. Add 1 c.c. of "qualitative uric acid reagent" (see p. 171). Then add 10 c.c. of saturated sodium carbonate solution. A pronounced blue coloration should be obtained. Repeat the reaction with 5 c.c. of urine.

11. Volumetric Determination of Uric Acid.—Transfer to a small weighed beaker exactly 500 mg. of pure uric acid (Kahlbaum's). Add 50 c.c. of a .4 per cent. lithium carbonate solution and 25-35 c.c. of distilled water. Stir until dissolved. When solution is complete, transfer the solution without loss of a single drop to a 500 c.c. volumetric flask. Fill to the mark with water and mix. This alkaline uric acid solution will usually keep without appreciable decomposition for 5-7 days.

From 25 c.c. of the alkaline uric acid solution prepare a dilute, acidified (and therefore more permanent) standard solution of uric acid according to the directions given on p. 169. Transfer this solution, 20 c.c. of which contains 1 mg. of uric acid, to a bottle, label, and preserve for use in connection with colorimetric determinations of uric acid.

Prepare .05 N potassium permanganate solution by dissolving 1.578 g. in water and diluting to one liter. (Glass stoppered flask necessary.) Standardize this solution (in terms of uric acid) as follows: Measure 50 c.c. of the lithium carbonate solution of uric acid into a beaker, add 20 c.c. concentrated sulphuric acid, stir, and titrate immediately with the .05 N potassium permanganate solution. The very first coloration extending throughout the liquid marks the end point of the titration. Calculate the

number of mg. of uric acid to which each cubic centimeter of permanganate is equivalent.

Measure out two 50 c.c. samples of the alkaline uric acid solution. Add equal volumes of water to each and then stir in 10 g. ammonium sulphate until dissolved. Add about 5 c.c. concentrated ammonium hydrate, cover, and let stand for twenty-four hours. Filter. Wash the precipitate back into the beaker with about 100 c.c. water. Add sulphuric acid and titrate, as when standardizing the potassium permanganate solution.

THE DETERMINATION OF URIC ACID IN URINE: To 150 c.c. fresh urine add 37.5 c.c. special ammonium sulphate solution.* Let stand for half an hour, decant, and unless perfectly clear filter through a dry filter. Measure out 125 c.c. of the filtrate (corresponding to 100 c.c. urine), add about 5 c.c. ammonia, and let stand for 48 hours. Filter and wash the precipitate five or six times with 10 per cent. ammonium sulphate solution. Wash the precipitate into a beaker by means of about 100 c.c. water. Add sulphuric acid and titrate. Calculate the twenty-four hour quantity of uric acid and of uric acid nitrogen.

12. New Colorimetric Method for the Determination of Uric Acid.—Because of the fading of the blue color produced by the action of the phosphotungstic acid reagent on uric acid, the colorimetric quantitative determination has now been revised so as to permit the use of the reagent previously used only for phenol determinations—the phenol and uric acid reagent. The revisions involved are quite extensive.

The new method is now as follows:

Transfer 2-5 c.c. of urine, according to concentration, to a 12 c.c. centrifuge tube, and add distilled water until the tube contains about 6 c.c. Add 5 c.c. of a silver lactate solution (silver lactate 5 per cent., lactic acid 5 per cent.), and stir the mixture thoroughly with a glass rod. Rinse off the rod with a few drops of distilled water. Centrifuge the counterbalanced tube for 2-3 minutes. Add a drop of the silver solution to the clear supernatant liquid; if a precipitate forms, add 2 c.c. more of the silver solution and centrifuge again; if no precipitate forms, pour off the liquid as completely as possible.

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* The solution contains 50 per cent. ammonium sulphate, 5 per cent. uranium acetate, and 5 per cent. acetic acid.

To the precipitate in the centrifuge tube add, from a buret, 4 c.c. of a 5 per cent. sodium cyanid solution (*poisonous—3 c.c. may be fatal dose*) and about 5 c.c. of water. Stir the mixture with a glass rod until a perfectly clear solution is formed. Rinse the rod, collecting the rinsings in a 100 c.c. graduated flask; pour the contents of the tube into the same flask; and rinse the tube twice with about 5 c.c. of water, which is also poured into the flask. Add water, to a volume of about 40 c.c., and 4 c.c. of uric acid-phenol reagent (see p. 171).

In another 100 c.c. flask place 20 c.c. of a standard uric acid solution (see p. 169) containing 1 mg. of uric acid; add 4 c.c. of cyanid solution, about 20 c.c. of water, and 4 c.c. of uric acid-phenol reagent. Then add 20 c.c. of 20 per cent. sodium carbonate solution to each flask. At the end of 20 minutes fill both flasks up to volume, and read against each other in a colorimeter. If a precipitate has formed, the solutions must be filtered or centrifuged before making the color comparison.

Set the standard uric acid solution at 20 m.m., and read it against itself several times before reading the unknown. Twenty divided by the reading of the unknown (in m.m.) gives the amount of uric acid (in mg.) present in the volume of urine taken for analysis.

When working with urines containing albumin, the albumin must be removed by coagulation with acetic acid and filtering before making the determination.

13. Creatinin.—Transfer to a small bottle about 3 g. dry picric acid. Add about 100 c.c. of urine, insert a cork, and shake continuously for ten minutes. Filter a portion of the mixture and transfer 5 c.c. of the filtrate to a bottle or flask (capacity not less than 250 c.c.).

Measure 5 c.c. of the original urine into another similar flask or bottle. Add to each, first 20 c.c. saturated picric acid solution, and then 5 c.c. 10 per cent. sodic hydrate. Let stand 5-10 minutes and add 200 c.c. tap water to each.

Remove by decantation the liquid from the bottle containing urine and picric acid, and to the sediment add about 25 c.c. water and 15 c.c. sodic hydrate (10 per cent.). Shake, let stand for a few minutes, and fill the bottle with tap water.

The substance in urine which is precipitated by picric acid and which gives a deep red color with alkaline picrate solutions,

is creatinin. No other known substance occurring in normal urine gives this color reaction. Therefore, on the basis of this reaction, it is easy to determine the creatinin quantitatively.

14. Quantitative Determination of Creatinin (*J. Biol. Chem., 17, 469*).—A suitable and convenient "creatinin reagent" is prepared by adding 75 c.c. sodic hydrate (10 per cent.) to a liter of saturated picric acid solution. The solution keeps well in tightly stoppered bottles.

By means of an Ostwald pipet transfer 1 c.c. of urine to a 100 c.c. volumetric flask. To another similar flask transfer 1 c.c. of a standard creatinin solution (1.61 g. creatinin zinc chlorid dissolved in a liter .1 N hydrochloric acid), 1 c.c. of which contains 1 mg. creatinin. To each flask add 20 c.c. of the alkaline picrate solution and let stand for ten minutes. Dilute to the mark with water and mix.

Read the standard against itself in the colorimeter at 20 m.m. until the correct value (20 m.m.) can be obtained several times in succession. The error in reading should not exceed .2 m.m. Rinse the right-hand cup and prism with the unknown, and determine its color in terms of the standard set at 20 m.m. Twenty divided by the reading gives the creatinin in milligrams in the quantity of urine taken (1-5 c.c.).

Calculate the total twenty-four hour creatinin.

15. Creatin.—Unless considerable meat or fish has been eaten the urine of normal adults contains only traces of creatin. Urines of children and of sick persons, particularly fever patients, appear, on the other hand, to contain relatively considerable quantities of creatin (.2 g. to 1 g. or more per day in fever patients).

Creatin is determined in such urines by first converting it into creatinin. This is done as follows:

Measure the urine (usually 1 c.c.) into a flask (capacity 300 c.c.) and add 20 c.c. saturated picric acid solution (not the creatinin reagent). Weigh flask and contents and add about 150 c.c. water. Boil gently for 45 minutes, then more rapidly until the original volume (determined by weighing) is obtained (a variation of 3 or 4 g. makes no appreciable difference). Cool. Add 1.5 c.c. 10 per cent. sodic hydrate, let stand 10 minutes, and com-

pare, as in the case of preformed creatinin, with the color obtained from 1 mg. creatinin.

Twenty divided by the reading gives the sum of the creatin and creatinin present.

Calculate the twenty-four hour quantity and subtract the preformed creatinin.

If an autoclave is available, the conversion into creatin can be made more rapidly.

Measure the urine (1 c.c.) into a 100 c.c. volumetric flask, and add 20 c.c. saturated picric acid solution. Cover the mouth of the flask with tinfoil, and heat in the autoclave at 115°-120° for 20 minutes. Cool, add 1.5 c.c. sodic hydrate, and finish the determination in the usual manner.

16. Hippuric Acid.—Take with the evening meal 2 g. of sodium benzoate, and collect the urine until next morning.

Evaporate to small volume and transfer with a little wash water to a small flask. Acidify strongly with sulphuric acid and put away for twenty-four hours. Filter and dry the precipitate, consisting of hippuric acid, uric acid, and other substances. Extract the hippuric acid with acetic ether. Set aside for spontaneous evaporation. Examine microscopically. Heat the dry substance in a dry tube, and note the odor of bitter almonds (benzaldehyd).

17. Determination of Inorganic Sulphates (*J. Biol. Chem., 1, 131*).—Measure into a small flask or beaker 25 c.c. of the ammonium sulphate solution in which the ammonia was determined (p. 21). Dilute with water to about 100 c.c. Add 10 c.c. 20 per cent. sodium chlorid solution (free from sulphate), 5 c.c. concentrated hydrochloric acid, and finally, drop by drop (without shaking), about 15 c.c. 5 per cent. barium chlorid solution. Let stand for one hour, and filter through a previously prepared Gooch crucible (consult the instructor). Wash about a dozen times with cold water, ignite, and weigh. From the weight of $BaSO_4$ obtained, calculate the ammonium sulphate, and compare with the weight of ammonium sulphate used in making up the original solution.

18. Determinations of Total Sulphates.—Repeat the determination, omitting the addition of sodium chlorid, and heating the

solution to boiling for 15 minutes before adding the barium chlorid.

Repeat the two sulphate determinations described, with 25 c.c. of urine, but omitting the use of sodium chlorid. Explain the results.

19. Determination of Total Sulphur (*Benedict, J. Biol. Chem., 6, 363; W. Denis, J. Biol. Chem., 8, 401*).—To 25 c.c. of urine contained in a porcelain evaporating dish (10-12 cm. diameter) add exactly 5 c.c. of a solution containing 25 per cent. copper nitrate, 25 per cent. sodium chlorid, and 10 per cent. ammonium nitrate. Evaporate to dryness on the water-bath. Then heat over a flame, gradually increasing the heat until the dish is red hot, and continue heating for 10 to 15 minutes. Allow to cool. Add 20 c.c. dilute hydrochloric acid and warm gently. Rinse into a flask or beaker by means of about 100 c.c. hot water. Heat to boiling, and add drop by drop 25 c.c. of a 10 per cent. barium chlorid solution. Filter, wash, ignite, and weigh.

20. Phosphates.—Add a few drops neutral calcium chlorid solution:

(a) to 5 c.c. 1 per cent. monopotassium phosphate solution,
(b) to 5 c.c. 1 per cent. disodium phosphate solution,
(c) to a mixture of the two phosphate solutions,
(d) to 5 c.c. turbid urine,
(e) to 5 c.c. clear urine,
(f) to 5 c.c. of urine after filtering off the precipitate obtained by the addition of a little ammonia. Explain the results.

21. Determination of Phosphates.—Standard Phosphate Solution.—Dissolve 4.39 g. pure monopotassium phosphate in water and dilute to 500 c.c. Each c.c. contains 2 mg. phosphorus. Label and preserve.

Standard Uranium Solution.—Dissolve 18 g. uranium acetate and 50 c.c. 50 per cent. acetic acid in water. Dilute to 500 c.c. If turbid, allow to settle (for a day or two), and remove the clear supernatant solution by means of a siphon.

Transfer 25 c.c. of the phosphate solution to a flask, add 5 c.c. special sodic acetate solution (containing 10 per cent. acetate and 3 per cent. acetic acid), heat to boiling, and add 15-20 c.c. of the clear uranium solution from a buret. Heat again to boiling, and

now add the uranium slowly until 2 drops of the phosphate uranium mixture when added (by means of a glass tube drawn out to a point like a pipet) to a minute pinch of powdered potassium ferrocyanid (on a white plate) gives a faint yet unmistakable brownish coloration.

Repeat until the exact titrating value of the uranium solution has been ascertained.

Calculate the value of the uranium solution in terms of phosphorus (P). Label and preserve.

Determination of Phosphates in Urine.—Measure 50 c.c. of urine into a flask, add sodic acetate, heat to boiling, and titrate with the uranium solution, exactly as in the case of the standard phosphate solution. After having found the approximate phosphate content by means of the preliminary titration, repeat, adding nearly all the required uranium solution at once to the hot urine, and finish by adding only a few drops at a time.

Calculate in terms of phosphorus and of phosphoric acid the phosphate content of the 24 hour urine under examination.

22. Acidity of Urine.—Titrate, with phenolphthalein as indicator, the acidity of the monopotassium phosphate solution used for standardizing the uranium solution.

Repeat after having added 5 c.c. of neutral calcium chlorid solution (2 per cent.).

Repeat after having added 5 c.c. of the calcium chlorid solution and 5 c.c. of the saturated neutralized potassium oxalate solution which was used in the "formol titration" of ammonia. Explain the results.

Titrate the acidity of 20 or 25 c.c. of urine (a) with and (b) without the addition of 5 c.c. of the neutralized oxalate solution. From the result obtained in (a), calculate the acidity of the twenty-four hour urine in c.c. of decinormal acid. Calculate also the condition of the phosphates in the urine, assuming that the acidity of normal urine is first of all due to acid phosphates.

23. Determination of Chlorids.—Standard Silver Nitrate Solution.—This is prepared by dissolving 23.94 g. silver nitrate per liter of solution (or 5.99 g. in 250 c.c.).

Standard Ammonium Sulphocyanate Solution.—Dissolve 6 g. of the salt in 800-900 c.c. water. Transfer 10 c.c. of the silver solution to a beaker or flask; add 50 c.c. water, 5 c.c. concen-

trated nitric acid, and 2 c.c. of saturated ferric ammonium sulphate solution. By means of a buret titrate the acidified silver solution with the sulphocyanate solution. On the basis of the result, dilute a part of the sulphocyanate solution so as to give 500 c.c. (or a liter) of a solution, 20 c.c. of which is exactly equivalent to 10 c.c. of the silver solution.

Each c.c. of the silver solution corresponds to 5 mg. chlorin (or to 8.23 mg. sodium chlorid).

The chlorin determination in urine is carried out as follows:

Pipet 10 c.c. of urine into a 100 c.c. volumetric flask, add 50 c.c. distilled water, 5 c.c. saturated ferric alum solution, and 5 c.c. concentrated nitric acid. Add 20 c.c. standard silver nitrate solution, fill up to the mark with distilled water, and shake. Filter through a dry filter into a dry beaker or flask. With a clean, dry pipet transfer 50 c.c. of the filtrate to another beaker, flask, or evaporating dish, and titrate in the same manner as when standardizing the silver solution.

Since the sulphocyanate solution is half as strong as the silver solution, and since only one-half of the surplus silver was taken for titration, 20 minus the sulphocyanate titration figure represents the silver nitrate which has combined with the chlorin of the urine to form silver chlorid. This figure multiplied by 5 or by 8.23 gives the chlorin or sodium chlorid (in milligrams) present in 10 c.c. of urine.

Calculate the twenty-four hour quantity.

24. Indican.—To 10 c.c. of urine add 2 c.c. of copper sulphate solution, 5 c.c. chloroform, and an equal volume (17 c.c.) of strong hydrochloric acid. Close the mouth of the tube with the thumb, and cautiously invert a few times.

The amount of indican present is proportional to the depth of color of the chloroform extract. This qualitative test is often made roughly quantitative by using the color of Fehling's solution as a standard.

25. Metabolism Experiments.—Weigh accurately a small, clean, dry flask. Pipet into it 25 c.c. of urine and weigh again. From the data obtained calculate the specific gravity of the urine.

Determine the specific gravity of the same sample of urine by means of an ordinary clinical areometer. Compare the results obtained, and explain how to standardize a clinical areometer.

Collect a full twenty-four hour quantity of urine, and in it determine the following: Volume, specific gravity, acidity, total nitrogen, urea, ammonia, uric acid, creatinin, chlorids, phosphates, sulphates, ethereal sulphates. Test qualitatively for indican.

For two days eat no meat, fish, eggs, milk, cheese, peas, or beans, and only a little bread. Eat much butter, potatoes, vegetables, starch preparations, fruit, and candy. Repeat all the determinations with the second twenty-four hour quantity.

For two days eat all the meat products you can, and collect the two twenty-four hour quantities of urine. In the second twenty-four hour quantity determine all the factors enumerated above. Tabulate and compare the results obtained in the three series of analyses.

Take 15 g. of sodium bicarbonate in divided doses, collect the twenty-four hour urine, and estimate the ammonia and acidity. Compare with the normal.

Two days later, beginning in the morning, take 5 g. ammonium chlorid in the course of the day. Determine the ammonia and acidity.

Eat much sweetbread, kidney, or liver, for one day; collect the urine for the whole twenty-four hours, and estimate the uric acid. Compare with the uric acid figures previously obtained. Explain.

PART VII

BLOOD

1. **Hemoglobin Crystals.**—Place a drop of defibrinated rat blood on a slide, add a drop or two of water, mix, and cover with a cover-glass. Sketch the crystals which separate after a few minutes.

2. **Hemoglobin (Reduced Hemoglobin).**—Add to dilute blood a few drops of strong ammonium sulphid, or one or two drops of freshly prepared Stokes' reagent.

Examine spectroscopically.

Stokes' reagent is a 2 per cent. solution of ferrous ammonium sulphate in 3 per cent. tartaric acid, to which is added ammonia until a clear solution is obtained. The ammonia should be added only to the amount of reagent immediately needed.

Shake the solution of hemoglobin with air, and note the rapid change to oxyhemoglobin. Change the same solution of oxyhemoglobin to hemoglobin, and reverse two or three times, and note the facility with which hemoglobin takes up and loses oxygen.

3. **Carbon monoxid hemoglobin.**—Pass a current of illuminating gas through a dilute oxyhemoglobin solution for a minute, and filter. Note the change of color. Try the effect on the solution of (1) ammonium sulphid, (2) Stokes' reagent, (3) potassium ferrocyanid, (4) shaking with air. Note the stability of the compound.

Examine spectroscopically.

4. **Methemoglobin.**—Add to dilute defibrinated blood (1:15) two drops of a freshly prepared solution of sodium nitrite. Note the change. What is the effect produced by the addition of reducing agents?

5. **Hematin.**—Hemolyze a small quantity of blood and add dilute hydrochloric acid cautiously till a precipitate occurs.

Acidify strongly with hydrochloric acid. Note color (acid hematin). Then add sodium hydrate till strongly alkaline. Note color (alkaline hematin). To the alkaline solution add a few drops ammonium sulphid and warm gently. Note color (reduced hematin or hemochromogen).

6. **Hemin Crystals—Teishmann's Test.**—Place a bit of powdered dried blood on a glass slide, add a minute crystal of sodium iodid and two drops of glacial acetic acid. Cover with a cover-glass and warm gently over a flame until bubbles appear. Describe the crystals which separate.

7. **Sugar in Blood.**—Mix 25 c.c. of fresh blood with 150 c.c. water and heat to boiling. Add 10 c.c. "colloidal iron" solution, shake, and filter. Concentrate the clear filtrate to about 25 c.c. Test for sugar.

8. **Fibrinogen.**—Allow about 6-8 volumes fresh blood to run from the animal into 1 volume of a 1 per cent. potassium oxalate solution (why?). Allow to stand over night in the cold room, and siphon off the clear plasma. With the solution so obtained make the following experiments:

Dilute 10 c.c. with 20 c.c. of distilled water and divide into 3 equal portions. To one add a little dilute (1 per cent.) calcium chlorid solution. To the second add a few drops of blood serum (why?). Place the three tubes in a beaker of water heated to 40° and observe the time of clotting.

PART VIII

MILK

Determine the specific gravity as in the case of urine.

1. **Determination of Total Nitrogen.**—Transfer 25 c.c. milk to a 100 c.c. volumetric flask. Fill to the mark with water, mix, and determine the total nitrogen in 1 c.c. Calculate the total protein content of the milk by multiplying its nitrogen with the factor 6.25.

2. **Determination of Casein.**—Transfer 50 c.c. of the diluted milk to another 100 c.c. flask, and carefully precipitate the casein by the addition of dilute acetic acid and gentle shaking. Make up to volume (100 c.c.) with water. Centrifuge a portion, and determine the nitrogen in 5 c.c. of the clear liquid.

Calculate the total nitrogen (and protein), making due allowance for the dilutions, and subtract from the total protein found in the preceding experiment. The difference represents casein.

3. **Determination of Lactose.**—Precipitate the proteins, etc., by acid mercuric nitrate. This reagent is made by dissolving mercury in double its weight of concentrated nitric acid and diluting with an equal volume of water.

To 50 c.c. of milk in a 100 c.c. measuring flask add 1 c.c. of the nitrate solution, fill up to the 100 c.c. mark with water, and agitate. Filter through a dry filter, and determine the sugar in the filtrate by means of the polariscope. The result will be a trifle low (about 2 per cent.) on account of the volume occupied by the curd.

In the case of mothers' milk it is often inconvenient to try to secure 50 c.c. In such cases 10 c.c. and a 25 c.c. volumetric flask may be used; count the drops necessary to give 1 c.c. of the nitrate solution, and add one-fifth of that number of drops (i. e., .2 c.c.); then fill up to the mark, filter, and polarize.

4. Determination of Fat.—Measure out 17.6 c.c. of thoroughly mixed milk into a Babcock flask. Add 17 c.c. sulphuric acid (sp. gr. 1.82) and mix thoroughly, with gentle turning and shaking, until all the precipitated proteins have dissolved. Rotate in the centrifuge for 3 minutes. Add hot water up to the beginning of the graduations in the neck of the flask, and rotate for 1 minute. The graduations read in per cent. of fat.

PART IX

BONE

Weigh a piece of clean, raw bone on the laboratory scales. Immerse in about 10 times its weight of 10 per cent. hydrochloric acid in a flask. If any gas is evolved, determine what it is.

After 48 hours dilute the volume of the solution and what remains of the bone to a definite volume in a cylinder. Mix so as to get the solution uniform in composition.

Pipet out 25 c.c. of the solution, neutralize with sodic hydrate, using congo red paper as indicator, and determine the phosphates. Repeat.

Calculate the tricalcic phosphate corresponding to the phosphoric acid found.

Taking other portions of the original solution, demonstrate experimentally that all the calcium in the bone can not be precipitated together with the phosphoric acid present.

In what form is this excess of calcium present in bone?

Examine the insoluble substance left in the hydrochloric acid solution. What is the substance? Prepare a "gelatin" solution from it.

PART X

BILE

1. **Character of Bile.**—Determine the specific gravity, taste, odor, color, consistency, reaction, of the bile supplied.

Test for coagulable protein.

2. **Bile Salts.**—Mix 250 c.c. ox-bile with one-fourth its volume of bone-black, and evaporate nearly to dryness on the water-bath. Cool, transfer the residue to a flask, and extract with 200 c.c. of alcohol over night. Filter, and evaporate the filtrate to dryness on the water-bath. Dissolve the residue in absolute alcohol, and filter into a dry flask. Add anhydrous ether till permanent cloudiness develops. Place in the cold room to crystallize. Filter. Describe the crystals.

3. **Pettenkofer's Test for Bile Salts.**—Mix a little bile with 2 or 3 drops of 10 per cent. solution of cane sugar. Place in a test tube some concentrated sulphuric acid. Incline the tube containing the sulphuric acid, and pour the bile solution slowly down the side of the tube so that it forms a layer above the sulphuric acid.

4. **Gmelin's Test for Bile Pigments.**—Put 5 c.c. of nitric acid, containing some nitrous acid, in a test tube, and introduce on top of it (pipet) about 5 c.c. of diluted bile. Note what occurs. Study the delicacy of the reaction with very dilute solution of bile.

5. **Test for Bile in Urine.**—The presence of bile in human urine is usually indicated by its color and the color of the foam. In making the nitric acid test for albumin the presence of bile is also revealed by a series of colored rings (green, blue, violet, red, and yellowish-red).

A similar series of colors is occasionally obtained from urines which have been preserved with thymol. This is one of the objections to this otherwise excellent preservative.

To 10 c.c. of urine add a few drops of calcium chlorid solution and a few drops of 10 per cent. sodic hydrate. Filter. Remove the filter paper from the funnel, open it, and drop 1 or 2 drops of concentrated nitric acid on the sediment. In the presence of (human) bile the usual, characteristic series of colored rings is obtained.

SUPPLEMENT

URINE

Qualitative Test for Acetone in Urine.—Clinicians seldom differentiate between acetone and diacetic acid, and the "acetone tests" which they use are tests for diacetic acid rather than for acetone. (See p. 133.)

In the qualitative test for acetone, as for its quantitative determination, the acetone is first removed from the urine by means of an air current, just as in corresponding determinations (and tests) for ammonia.

In the large test tube used for the colorimetric determination of ammonia place first 5 c.c. of urine and 1-2 drops dilute acid (HCl or H_2SO_4). Then insert the rubber stopper carrying the absorption tube, etc., place the test tube in a beaker of lukewarm water (35-40° C.), and aspirate the volatile acetone by means of a moderately rapid air current into a test tube containing 5 c.c. distilled water and 5 c.c. Scott-Wilson reagent. If acetone is present, even if only in minute traces, the solution becomes turbid. If the amount of acetone obtained is extremely small the turbidity may not appear for 5-10 minutes.

The Scott-Wilson reagent for acetone, which is used for qualitative tests as well as for quantitative determinations, is most conveniently prepared as follows:

To 10 g. of mercuric cyanid dissolved in 600 c.c. of water add a cooled solution of 180 g. of sodium hydroxid in 600 c.c. of water. Transfer this mixture to a heavy walled glass jar, and to it add 2.9 g. of silver nitrate dissolved in 400 c.c. of water. The silver solution should be added in a slow stream, and the addition must be accompanied by constant and exceedingly vigorous stirring with a heavy glass rod. If properly made, the silver dissolves completely, giving a clear solution which is at once available for use. If the solution is turbid, it should be set aside to settle for three or four days and the clear supernatant liquid removed by means of a siphon.

In the clear reagent a new sediment gradually forms, so that the solution deteriorates slowly and after a few months is not serviceable for quantitative work, though still good for qualitative tests.

Titration of Acetone and Preparation of Standard Acetone Solutions.—Standard solutions of iodin, sodium thiosulphate, and potassium permanganate are needed in this work, the latter being used only as a basis for making the other two accurate. The .05 N permanganate solution used for the titration of uric acid (see p. 91) may be used for this purpose.

Iodin.—Weigh roughly 10-12 g. of potassium iodid in a beaker and add 50 c.c. of water. Weigh out 6.4 g. iodin in a small beaker covered with a watch glass and add this to the potassium iodid solution. Stir until the iodin is dissolved and then transfer the resulting solution to a 500 c.c. volumetric flask. Dilute to the mark with water and mix.

Sodium Thiosulphate.—Weigh out 24.85 g. of the salt ($Na_2S_2O_3 + 5H_2O$), dissolve in water, transfer to a 500 c.c. volumetric flask, fill to the mark with water, and mix.

The two solutions thus prepared should be approximately tenth normal. Their relative values are determined by titration as follows:

Pipet 20 c.c. of the iodin solution into a flask (capacity 500-600 c.c.) and add about 100 c.c. water. From a buret, run in the thiosulphate solution until the reddish-brown iodin color has faded to a faint straw yellow. Now add a few drops of starch paste (see p. 57) and continue the titration till the blue iodid of starch color disappears. The end point of this titration is very sharp.

The value of the thiosulphate solution is now determined as follows:

Weigh roughly 2 g. potassium iodid, transfer to a flask, and dissolve in about 150 c.c. water. Add 5 c.c. diluted hydrochloric acid (1-5) and 50 c.c. .05 N potassium permanganate solution.

The permanganate sets free an equivalent quantity of iodin according to the following equation:

$$2KMnO_4 + 10KI + 16HCl = 12KCl + 2MnCl_2 + 8H_2O + 5I_2$$

The iodin thus liberated is then titrated with the sodium thiosulphate solution in the same manner as the original iodin solution.

From the relative values of the iodin, the thiosulphate, and the permanganate solutions, the exact values of the first two (in terms of tenth normal solutions) are calculated.

Standard Stock Solution of Acetone.—Add about 1 c.c. of *pure* acetone (from the bisulphite compound) to water in a one liter volumetric flask, dilute to the mark, and mix. The titration of acetone with iodin is based on the fact that in alkaline solutions the acetone is converted into iodoform. Several reactions are involved in this process:

Iodin is converted into hypo-iodite,

1. $I_2 + 2KOH = KOI + KI + H_2O$.

Hypo-iodite is then slowly converted into (useless) iodate,

2. $3KOI = KIO_3 + 2KI$.

The hypo-iodite converts acetone into iodoform and acetic acid,

3. $3KOI + CH_3COCH_3 = CH_3\ COCI_3 + 3KOH$.
4. $CH_3\ COCI_3 + KOH = CH_3COOK + CHI_3$.

On acidifying, after the iodoform has been formed, the surplus iodin present as hypo-iodite (and iodate) is set free, and can then be titrated with the standard sodium thiosulphate solution as described above.

Each molecule of acetone uses up three molecules of hypo-iodite, and as each molecule of the latter is formed at the expense of two atoms of iodin, six atoms of iodin correspond to one molecule of acetone. One c.c. of .1 N iodin solution corresponds therefore to .968 mg. acetone. Because of the iodate formation a considerable excess must be added.

The titration of the acetone solution is carried out as follows:

Transfer 25 c.c. of the stock acetone solution to a flask, add 150-200 c.c. water, then 50 c.c. of the standardized iodin solution, and 10 c.c. strong sodic hydrate (40 per cent.). Let stand with occasional shaking for 5 minutes. Add 18 c.c. concentrated hydrochloric acid, and titrate the liberated excess of iodin with the standard thiosulphate solution.

If the standard solutions are exactly tenth normal, subtract the volume of thiosulphate employed from the volume of iodin solution taken, and multiply the remainder (in c.c.) with .968 to obtain the acetone content (in mg.).

Calculate the acetone content of the stock solution (in mg. per c.c.). Transfer to a distilling flask as much of it as contains exactly 50 mg. of acetone. Add water enough to make a volume of 500-600 c.c., and distill with vigorous cooling of the condenser. The receiver should be a large flask (750-1000 c.c.) containing about 250 c.c. approximately normal sulphuric acid. Boil for 20-30 minutes, or until at least 150 c.c. of distillate has gone over. Transfer this distillate to a volumetric (liter) flask and dilute to the mark with water. Ten c.c. of the acetone solution so obtained contains .5 mg. acetone. This solution, as well as the original stock solution, should be kept in a well stoppered bottle. The sulphuric acid present in the dilute standard acetone solution is added to prevent polymerization.

Preparation of Standard Acetone Solutions from the Acetone Bisulphite Compound.—A standard acetone solution can be prepared without distillations from the "acetone sulphite" used in photography as follows:

Transfer 2.5 g. of the powder to a volumetric (1,000 c.c.) flask by means of a little water (50 c.c.), and fill up to the mark with dilute (1 in 5) hydrochloric acid. Transfer 25 c.c. of the solution to a flask. Add 20 c.c. tenth normal iodin, let stand for five minutes, and titrate the surplus iodin with tenth normal thiosulphate solution. This titration gives the SO_2 or the sodium bisulphite content.

To another 25 c.c. of the acetone solution add 50 c.c. tenth normal iodin, let stand five minutes, then add 10 c.c. strong sodic hydrate, followed after five minutes by 18 c.c. concentrated hydrochloric acid. Titrate the liberated iodin with thiosulphate. From the 50 c.c. of iodin taken subtract (a) the figure of the last thiosulphate titration and (b) the iodin corresponding to the SO_2. The remainder corresponds to the acetone. From the standardized stock solution prepare the more dilute standard solution (5 c.c. or 10 c.c. of which should contain exactly half a mg. of acetone).

If the "acetone sulphite" is not available, acetone sodium bisulphite is easily prepared by slowly adding (with stirring) two-thirds volume of ordinary acetone to one volume (100 or 200 c.c.) of saturated sodium bisulphite solution (freshly prepared and filtered). The precipitate should be freed as completely as possible from the mother liquor by filtering on a Buchner funnel with suction. Then wash rapidly two or three times with alcohol. Let dry in the open air for two or three days. Sieve to make the preparation uniform, and preserve in glass stoppered vessel.

Quantitative Determination of Acetone in Urine.—To about 1 c.c. of 10 per cent. sulphuric acid in a large test tube add enough urine (.5 to 5 c.c.) to give about .5 mg. of free acetone (.3-.7 mg.). Connect the test tube, as in ammonia determinations, with a second test tube containing 10 c.c. of fresh approximately 2 per cent. sodium bisulphite solution. Warm the first test tube to 35-40° C., as in the qualitative test for acetone and aspirate the acetone into the bisulphite solution by means of a moderate air current (time about 10 minutes). Transfer the sulphite-acetone solution to a 100 c.c. volumetric flask together with distilled water enough to make 50-60 c.c. To each of two other 100 c.c. flasks add 10 c.c. of the standard acetone solution containing .5 mg. acetone, add 10 c.c. of the 2 per cent. bisulphite solution, and dilute with distilled water to 50-60 c.c.

To each of the three flasks add 15 c.c. (clear) Scott-Wilson reagent, and immediately (before turbidity formation) dilute with distilled water to the mark, mix, and let stand for 12-15 minutes. Read the turbid contents of one of the standard acetone suspen-

sions against itself in the Duboscq colorimeter. The best source of light for these comparisons is diffuse daylight coming through an opening (about 25 cm. square) cut through the shade of a (north side) window. The colorimeter metal screen must also be used. The instrument must be adjusted until 20 m.m. of the two suspensions are equal. Now replace the contents of one of the colorimeter cups with the contents of the second standard suspension, and the other with the unknown acetone mercury suspension obtained from the urine, and make the turbidity comparison in the same manner as colorimetric comparisons—setting the standard at 20 m.m.

Twenty multiplied by .5 and divided by the reading of the unknown (in m.m.) gives the acetone content (in mg.) of the volume of urine taken for the analysis.

Qualitative Test for Diacetic Acid (in traces).—To 5 c.c. of urine in a test tube add 1-2 c.c. dilute acetic acid (10 per cent.) and a small crystal of sodium nitroprussid. Shake a few times to dissolve the salt, then add an excess of concentrated ammonia (2-3 c.c.), and mix. A violet color indicates diacetic acid.

Gerhardt's Ferric Chlorid Test for Diacetic Acid.—This test is useful for showing the presence in urine of relatively large amounts of diacetic acid. It is made as follows: To 5 c.c. of urine in a test tube add ferric chlorid solution (10 per cent.), drop by drop. At first a white precipitate of ferric phosphate is obtained, then, as the addition of the reagent is continued, a dark red color is produced if diacetic acid is present (in more than traces).

A number of substances used as drugs, such as salicylic acid, phenacetin, etc., give a similar reaction. If confusion due to such drugs is to be suspected, boil the deep red solution for 2-3 minutes. If the color is due to diacetic acid, it should disappear during the boiling and not reappear on cooling. The disappearance is due to the destruction by boiling of the unstable diacetic acid.

Quantitative Determination of Diacetic Acid (and Acetone).—Acetone urines contain from two or three to nine or ten times as much aceto-acetic acid as acetone. In strictly fresh urines the latter proportions prevail; but the older the urine the greater

becomes the relative proportion of acetone, because of the spontaneous decomposition of the aceto-acetic acid. Urines giving a strong ferric chlorid reaction usually contain more than .5 mg. of aceto-acetic acid per cubic centimeter, and must be diluted so that an appropriate fraction of 1 c.c. (of the original urine) can be taken for a determination.

The amount of urine taken should yield approximately .5 mg. of acetone (from .3 to .7 mg.). Transfer this amount of urine to a large test tube containing 1 c.c. of 10 per cent. sulphuric acid, and connect with a second test tube containing 10 c.c. of 2 per cent. sodium bisulphite solution. Immerse the test tube containing the urine in a beaker of boiling water and the second test tube in cold water, then pass through an *extremely* slow air current for ten minutes. Increase slightly the speed of the air current and continue the aspiration for another five minutes. The aceto-acetic acid plus acetone is thus transferred, in the form of acetone, to the bisulphite solution. Rinse the solution into a 100 c.c. volumetric flask, and determine the acetone exactly as in the determination of the preformed acetone.

One mg. of acetone is equivalent to 1.8 mg. of aceto-acetic acid. From the "total acetone" of the 24-hour quantity of urine is subtracted the total preformed acetone, and the remainder multiplied by 1.8 gives the aceto-acetic acid.

Determination of Beta-oxybutyric Acid in Urine.—The method described below was at first thought by its authors (Folin and Denis) to give strictly all the beta-oxybutyric acid present in urine. But it now appears that the yield is only 85-95 per cent., just as in the original method of Shaffer.

The urine is diluted from 10-100 times, depending on how much of the substance is present. The ammonia content of the urine is the best index as to how much urine is required to yield the desired amount of beta-oxybutyric acid (1.5-3.5 mg.). The ferric chlorid test for diacetic acid is also helpful, but without considerable experience only a preliminary determination can give the desired information.

Measure diluted urine, equivalent to 1.5-3.5 mg. of beta-oxybutyric acid, into a 500 c.c. Kjeldahl flask, add a little dilute sulphuric acid (5 c.c.), and water enough to make a volume of about 150 c.c. Boil the mixture gently for ten minutes (to drive off the preformed acetone and the diacetic acid), then add to the solution

(with a cylinder) 25 c.c. of a solution containing 1 per cent. potassium dichromate and 35 per cent. sulphuric acid, and connect the flask, in the usual manner, with a condenser by means of a specially treated rubber stopper.

The rubber stopper should be boiled twice for an hour in 10 per cent. sodic hydrate solution (or better, heated in an autoclave in the same solution for half an hour at 130-140° C.), and then thoroughly washed. It is also necessary to wrap the stopper thoroughly in tin foil during the distillation, so as to exclude the volatile sulphur impurities which otherwise are given off and interfere with the subsequent turbidity formation.

Distill very slowly, for one and one-half hours, collecting the distillate (about 100 c.c.) in another 500 c.c. Kjeldahl flask, previously charged with about 100 c.c. of water.

To the distillate add a small amount sodium peroxid (2 g.), and redistill by ordinary rapid boiling. Collect this final distillate in a 100 c.c. volumetric flask (or cylinder). About 80 c.c. should be obtained.

Dilute this distillate to the 100 c.c. mark with distilled water and mix. Transfer from 25 to 50 c.c. into a 100 c.c. volumetric flask, and determine the acetone content by the turbidity method, as in the case of the two preceding (acetone) determinations. No bisulphite is used in this case to hold the acetone, and none should therefore be added to the standard. Each milligram of acetone obtained corresponds to 1.78 mg. of beta-oxybutyric acid.

Shaffer's Short Method for the Determination of Beta-oxybutyric Acid.—To 50 c.c. of urine add 100 c.c. of water, then 50 c.c. of basic lead acetate solution (Goulard's Ext. U.S.P.), and stir. Add 50 c.c. approximately normal NaOH and stir again. Filter. A clear filtrate containing but traces of lead or glucose is usually obtained, even though the original urine contained considerable quantities of sugar. Traces of sugar do not interfere with the determination.

Introduce 50 c.c. of the filtrate into a 500 c.c. Kjeldahl flask previously marked at the level of 100 c.c. with a "glass pencil." Add 25 c.c. of water and 50 c.c. of half concentrated sulphuric acid. The latter, if freshly prepared by mixing with water (1 : 1), must be cooled before it is used.

Connect the Kjeldahl flask with a dropping funnel and with a

condenser. Distill off about 25 c.c., collecting the distillate in another Kjeldahl flask.

The first distillate thus obtained contains the preformed acetone, as well as the acetone derived from the aceto-acetic acid of the urine. By adding to it 5 c.c. of strong alkali and redistilling, for 10 minutes, this acetone is obtained, in the second distillate, free from impurities, and can be titrated with iodin and thiosulphate.

After replacing the Kjeldahl flask used as a receiver with another one, the distillation of the urine filtrate is continued, while adding slowly (about 15 drops per 10 seconds) a .2 per cent. potassium bichromate solution.

During this distillation the volume in the distilling flask should be kept at approximately 100 c.c. (i. e., at the level indicated by the pencil mark). This is readily accomplished by regulating the speed of the distillation so that it just about equals the speed with which the bichromate solution is added. The speed of the oxidation is much greater with increasing concentration of sulphuric acid. With too great concentration of the acid, however, when the volume approaches a level of about 70 c.c., the oxybutyric acid is in part converted into crotonic acid, and thus escapes oxidation to acetone.

The bichromate solution is added only so fast as to maintain a *very slight* excess; the blue green color should largely predominate in the boiling mixture. Occasionally it may be necessary to interrupt the addition of bichromate for a few minutes, but the volume in the distilling flask should not be allowed to sink below 85 or 90 c.c. The addition of bichromate should be continued until (at the concentration of acid used) no more appears to be converted into the green chromium salt. From 50 c.c. to 100 c.c. bichromate solution (= .1 g.-.2 g. $K_2C_2O_7$) is usually required for each distillation. The addition (and distillation) lasts 20-30 minutes.

The distillate obtained must be redistilled, after the addition of 5 c.c. strong alkali and about 20 c.c. of 30 per cent. hydrogen peroxid. This final distillation need not last more than 10 minutes. The distillate thus obtained is titrated in the usual manner (p. 127) with iodin and thiosulphate. The yield of acetone obtained is about 90 per cent. of the theoretical amount when working with solutions of pure beta-oxybutyric acid. A correction of 10 per cent. should therefore be added to the results obtained.

Slightly higher results (93-94 per cent.) may be obtained by a very slow addition of the bichromate, and a considerable prolongation of the distillation period, but since the theoretical amounts of acetone cannot be obtained the advantage so gained is doubtful.

Colorimetric Method for the Determination of Phenols in Urine.—The phosphotungstic phosphomolybdic reagent described in connection with the colorimetric determination of uric acid (p. 171) was originally devised as a reagent for phenols, and is serviceable for the determination of phenols in urinary filtrates from which the uric acid has been removed.

Transfer 10 c.c. of ordinary, or 20 of very dilute, urine to a 50 c.c. volumetric flask. Add acid silver lactate solution * (from 2 to 10 c.c.) until no more precipitate is obtained, then add a few drops of colloidal iron, and shake. Fill to the mark with distilled water, shake again, and filter. By means of this precipitation uric acid and traces of proteins are quantitatively removed. Transfer 25 c.c. of the filtrate to a 50 c.c. volumetric flask, and to it add a sufficient quantity of saturated sodium chlorid solution (containing 10 c.c. of strong hydrochloric acid per liter) to precipitate all the silver. Fill to the mark with distilled water and filter.

To determine "free" (non-conjugated) phenols, place 20 c.c. of this filtrate in a 50 c.c. flask, and treat with 5 c.c. of the phosphotungstic phosphomolybdic acid reagent and 15 c.c. of saturated sodium carbonate solution. After diluting to volume with lukewarm water (30-35° C.) and allowing to stand for twenty minutes, read the deep blue solution in a Duboscq colorimeter against a standard solution of phenol.

To determine total (free and conjugated) phenols, transfer 20 c.c. of the same filtrate to a large test tube, add ten drops of concentrated hydrochloric acid, and cover the test tube with a small funnel. Heat rapidly to boiling over a free flame, and then place in a boiling water-bath (usually a tall beaker) for ten minutes. At the end of this time remove the tube, cool, and transfer the contents to a 100 c.c. volumetric flask. Add 10 c.c. of the phosphotungstic phosphomolybdic reagent and 25 c.c. of sat-

* This solution consists of a 5 per cent. silver lactate solution in 5 per cent. lactic acid.

urated sodium carbonate solution. Make up to volume, shake, and let stand for 20 minutes. Read against a standard solution of phenol.

The standard is a solution of pure phenol in .01 N HCl, containing .5 mg. of the former substance in 5 c.c. To 5 c.c. of the standard solution in a 100 c.c. flask add 10 c.c. of the reagent and 25 c.c. of saturated sodium carbonate solution. Fill up to the mark with water (at about 30° C.), and make the color comparison in the usual manner, setting the standard at 20 m.m. As phenol is an exceedingly hygroscopic substance, it is necessary to standardize the solution by means of the iodometric titration.

This titration is carried out as follows: Make a phenol solution in .1 N HCl, containing 1 mg. of crystallized phenol per c.c. Transfer 25 c.c. of the phenol solution to a 250 c.c. flask, add 50 c.c. .1 N sodic hydrate, heat to 65° C., add 25 c.c. .1 N iodin solution, stopper the flask, and let stand at room temperature thirty to forty minutes. Add 5 c.c. of concentrated hydrochloric acid and titrate excess of iodin with .1 N sodium thiosulphate solution. One c.c. of .1 N iodin solution corresponds to 1.567 mg. of phenol. On the basis of the results, dilute the phenol solution so that 10 c.c. contains 1 mg. of phenol.

Because of the red precipitate in the solution it is rather difficult to see the end point of the titration. For those who have not had much experience it may be advisable to dilute the solution to a definite volume (after adding the hydrochloric acid), then to filter, and to titrate a portion of the filtrate as recommended by Sutton; with a little practice, however, the titration can be made without this procedure.

Quantitative Determination of Hippuric Acid in Urine.—In this method the hippuric acid is first hydrolyzed and the resulting benzoic acid is extracted with chloroform and the chloroform solution is titrated with standard alcoholic sodic hydrate.

Transfer 100 c.c. of urine to an evaporating dish, add 10 c.c. 5 per cent. sodic hydrate solution, and evaporate to dryness on the water-bath. Rinse the residue into a 500 c.c. Kjeldahl flask by means of 25 c.c. of water and 25 c.c. concentrated nitric acid. Add .2 g. copper nitrate, a couple of pebbles to prevent bumping, and boil very gently over a microburner for four and one-half hours. During this boiling a miniature Hopkins' condenser (made from a large test tube) is kept within the neck of the boiling flask to prevent loss of benzoic acid which is volatile with steam.

After cooling rinse the condenser with 25 c.c. water, and transfer the contents of the flask to a separatory funnel (capacity 500 c.c.). Rinse the flask with 25 c.c. water, thus making the total volume in the separatory funnel 100 c.c.

Add to this solution 55 g. of ammonium sulphate, shake until dissolved, and extract with neutral (freshly washed) chloroform four times, using 50, 35, 25, and 25 c.c. of chloroform respectively. Collect the chloroform extracts in another separatory funnel, and wash this by shaking with 100 c.c. saturated solution of pure sodium chlorid, to each liter of which has been added .5 c.c. concentrated hydrochloric acid.

Draw off the chloroform which contains the benzoic acid into a dry flask, and titrate with a dilute standardized sodium alcoholate solution and 4-5 drops of phenolphthalein as indicator. The first distinct coloration diffusing through the whole liquid is taken as the end point without regard to subsequent fading.

The sodium ethylate solution is made by dissolving from 1.8 g. to 2.3 g. metallic sodium in absolute alcohol and diluting to a liter with absolute alcohol. It is standardized against chloroform solutions of benzoic acid.

One cubic centimeter of .1 N alcoholate corresponds to 1.22 mg. benzoic acid or 1.79 mg. hippuric acid.

Turbidity Method for the Determination of Albumin in Urine.—To about 75 c.c. of water in each of two 100 c.c. volumetric flasks add 5 c.c. of a 25 per cent. solution of sulphosalicylic acid. To one flask add 5 c.c. of a standard protein solution, prepared as described below, and containing 10 mg. of albumin. To the other add the albuminous urine 1 c.c. at a time (by means of an Ostwald pipet) until the turbidity obtained seems to be reasonably near that of the standard. Fill the two flasks up to the mark with water, cautiously inverting a few times to secure mixing. The standard must invariably first be read against itself to secure the adjustment of the colorimeter (and of the eye). Then replace the contents of one of the Duboscq colorimeter cups by the suspension of the unknown, and make the turbidity comparison in the usual manner.

Set the standard containing 10 mg. of protein at 20 mm. The unknown must not read less than 10 nor more than 30 mm. Dividing 200 by the product of the reading of the unknown and

the number of cubic centimeters of urine taken, gives the albumin in milligrams per cubic centimeter of urine.

It is very important not to shake the albuminous suspensions in the volumetric flasks because of the tendency of the precipitate to agglutinate. The preliminary mixing must therefore be accomplished by means of a few gentle inversions.

The standard protein solution is prepared from fresh blood serum free from hemoglobin. For the preparation of this serum either slaughter house or normal human blood may be used. The so-called blood serum sold for the preparation of bacteriological culture media should be avoided, as it is usually several days old and is frequently partially decomposed. The dried preparations of "blood albumin" listed by chemical dealers are also not satisfactory for the preparation of standard solutions. To prepare the standard, dilute 25-35 c.c. of serum with a 15 per cent. solution of chemically pure sodium chlorid to about 1500 c.c. Mix and filter. By means of nitrogen determinations ascertain the protein content of the filtrate (protein = N X 6.25) and on the basis of the figure obtained, dilute the solution with 15 per cent. sodium chlorid solution so that it contains 2 mg. of protein per cubic centimeter. Sodium chlorid in the concentration mentioned is fairly effective as a preservative. Nevertheless it is best to saturate the standard albumin solution with chloroform (20 c.c.).

The above method is not applicable to urines which are very deeply colored with blood or bile pigments. The method is of course applicable to other albuminous fluids than urine, as, for example, exudates, transudates, and the cerebrospinal fluid.

Gravimetric Method for the Determination of Albumin in Urine.—The method is as follows: Pipet 10 c.c. of urine into an ordinary conical centrifuge tube, which has been previously weighed; add 1 c.c. of 5 per cent. acetic acid, and let stand for fifteen minutes in a beaker of boiling water. At the end of this time remove the tube from the water-bath and centrifuge for a few minutes. Pour off the supernatant liquid, stir up the precipitate in the tube with about 10 c.c. of boiling .5 per cent. acetic acid, and again centrifuge. Remove the supernatant liquid from the precipitate in the tube and wash once more, this time with 50 per cent. alcohol. After centrifuging and pouring off the supernatant alcohol, place the tube for two hours in an air bath at 100-110°, then cool in a desiccator, and weigh.

McCrudden's Method for the Determination of Calcium and Magnesium in Urine (*J. Biol. Chem., 7, 82 and 10, 187*).—If the urine is alkaline, make it neutral or slightly acid to litmus. Filter. Transfer 200 c.c. of the filtered urine to a small flask. Make just alkaline with concentrated ammonium hydrate and then just acid with (concentrated) hydrochloric acid. The cloud of phosphates forming in alkaline urine may be used as a guide in the process of acidifying the urine. Cool for a few minutes in running water. Add 10 drops of concentrated hydrochloric acid and 10 c.c. of 2.5 per cent. oxalic acid. Now add 8 c.c. of 20 per cent. sodic acetate solution, stopper, and shake vigorously and continuously for about ten minutes. Filter on a small ash free filter paper and wash free from chlorids with .5 per cent. ammonium oxalate solution. Transfer the filter and precipitate to a weighed platinum crucible, dry over a small flame, and then heat in the blast lamp to constant weight, thus transforming the calcium oxalate to calcium oxid. Cool in a desiccator and weigh.

In the combined filtrate and washwater the magnesium is determined as follows: Transfer the filtrate to a large porcelain dish, add 20 c.c. concentrated nitric acid, and boil down almost to dryness. When the residue is nearly dry and no more nitrous fumes are given off, add 10 c.c. concentrated hydrochloric acid and again boil down nearly to dryness. Dilute with water to a volume of almost 80 c.c., and with constant stirring add ammonia, drop by drop, until the mixture is alkaline to litmus paper. Then add 25 c.c. dilute ammonia (sp. gr. .96) slowly and with stirring, and set aside over night in a cool place. Filter on a small filter paper, and wash the precipitate with a dilute solution of alcohol and ammonia (1 volume of alcohol and 1 volume dilute ammonia mixed with 3 volumes of water). Wash until the filtrate is free from chlorids. Dry the filter and ignite in a weighed platinum crucible. Cool and weigh. The residue is $Mg_2P_2O_7$.

Method for the Determination of Sodium and Potassium in Urine.—Transfer 50 c.c. of urine to a platinum dish (capacity about 250 c.c.), evaporate to dryness, and then heat the residue, at first very cautiously, over a radial burner. Continue the heating at a barely perceptible dull red heat for one hour. Cool. Moisten the residue with 20 c.c. distilled water, evaporate to dryness, and heat as before for another hour. To the residue, which now should contain very little carbon, add 50 c.c. water and 5-6 drops con-

centrated hydrochloric acid. The mineral constituents are thus brought into solution. Add an excess of saturated barium hydroxid solution (i. e., to a distinctly alkaline reaction), heat to boiling, and filter on a Gooch crucible. Wash with hot water. The filtrate should now be substantially free from calcium, magnesium, phosphoric acid, and sulphuric acid, but does contain barium in addition to the sodium and potassium. Precipitate the barium by passing washed carbonic acid through the solution. Filter on another Gooch crucible and wash with a little cold water. Render the filtrate slightly acid to methyl orange (one drop), and evaporate to dryness in a previously weighed platinum dish. Heat the residue very gradually and carefully to a dull red heat for 10 minutes. Cool in a desiccator and weigh. The increase in weight gives the sodium and potassium as chlorids.

Dissolve the residue in a very small quantity of water and add a few drops dilute hydrochloric acid. Then add 10 per cent. chlorplatinic acid solution (4-5 times as much H_2PtCl_6 as the combined weight of the chlorids present), and evaporate at medium temperature, about 75° C., until the residue looks dry. Now add 95 per cent. alcohol, filter on a weighed Gooch crucible, and wash several times with 95 per cent. alcohol. Dry at 110° and weigh. The potassium chlorplatinate thus obtained multiplied by the factor .3056 gives the corresponding weight of potassium chlorid. The sodium chlorid is then obtained by subtracting the weight of the potassium chlorid from the weight of the combined chlorids.

In connection with this determination there are two fruitful sources of error: contamination of the chlorplatinate precipitate with ammonia (which, as ammonium chlorplatinate, gives too high results for potassium), and overheating during the ashing (which causes volatilization of the sodium chlorid). Loss of chlorids through overheating may be avoided by placing the platinum dish containing the dried urine on fragments of clay plate, or pieces of a broken evaporating dish, contained in a shallow iron dish (about 20 cm. in diameter) which is heated by means of a large size radial burner.

Indicator Method for the Determination of the Hydrogen-ion Concentration in Urine (*Henderson and Palmer, J. Biol. Chem., 13, 393*).—This determination, though simple in theory, is rather complicated in the practical execution because of the many standard solutions which must be used in each determination. A defi-

nite amount of a suitable indicator is added to a definite volume of urine (in 250 c.c. water) and to each of a series of solutions of known hydrogen-ion concentration (in 250 c.c. water). By matching of colors the solution having approximately the same hydrogen-ion concentration as the urine is thus found by inspection. The hydrogen-ion concentration is usually expressed, in accordance with the practice of Sörensen, in terms of its logarithm (P_H), with the negative sign left out.

The standard solutions employed by Henderson and Palmer can be prepared, perhaps most conveniently, from standardized solutions of sodic hydrate, phosphoric acid, and acetic acid. Half normal and tenth normal sodic hydrate, half normal and tenth normal phosphoric acid (figured as a dibasic acid), and half normal and tenth normal acetic acid, are the preliminary required stock solutions. The sodic hydrate should be standardized against accurate half normal hydrochloric acid, and should be as free as possible from carbonates. It must also be free from calcium or barium.

With accurate half normal sodic hydrate as the starting point, prepare a phosphoric acid solution which is somewhat stronger than half normal, taking account of only two of the three hydrogen atoms in the acid molecule. Titrate 25 c.c. of this acid to which has been added 10 g. neutral sodium chlorid, but no extra water. Use phenolphthalein as indicator. On the basis of the titration figures, prepare one or two liters of half normal, and one liter of tenth normal phosphoric acid. The acetic acid solutions are prepared in a similar manner.

Solution 1. (.1000 N Na_2HPO_4 P_H 9.27, used with phenolphthalein as indicator.)

To 200 c.c. .5 N phosphoric acid add 200 c.c. .5 N sodic hydrate, and dilute to 500 c.c.

Solution 2. (.0001 N NaH_2PO_4, .0480 N Na_2HPO_4, P_H 8.7, used with phenolphthalein as indicator.)

To 96.1 c.c. .5 N phosphoric acid add 96.05 c.c. .5 N sodic hydrate, and dilute to 500 c.c.

Solution 3. (.0001 N NaH_2PO_4, .0120 N Na_2HPO_4, P_H 8.0, used with alizarin red, neutral red, or phenolphthalein, as indicator.)

To 120.5 c.c. .1 N phosphoric acid add 120.25 c.c. .1 N sodic hydrate, and dilute to 500 c.c.

Solution 4. (.0166 N NaH_2PO_4, .0060 N Na_2HPO_4, P_H 7.48, used with alizarin red, or neutral red, as indicator.)

To 183.2 c.c. .5 N phosphoric acid add 174.9 c.c. .5 N sodic hydrate, and dilute to 500 c.c.

SOLUTION 5. (.0010 N NaH_2PO_4, .0060 Na_2HPO_4, PH 7.38, used with alizarin red, or neutral red, as indicator.)

To 65 c.c. .1 N phosphoric acid add 62.5 c.c. .1 N sodic hydrate, and dilute to 500 c.c.

SOLUTION 6. (.0010 N NaH_2PO_4, .0023 N Na_2HPO_4, PH 6.9, used with alizarin red, or neutral red, as indicator.)

To 28 c.c. .1 N phosphoric acid add 25.5 c.c. .1 N sodic hydrate, and dilute to 500 c.c.

SOLUTION 7. (.0009 N CH_3COOH, .0920 N CH_3COONa, PH 6.7, used with alizarin red as indicator.)

To 50.5 c.c. .1 N acetic acid add 46 c.c. .1 N sodic hydrate and dilute to 500 c.c.

SOLUTION 8. (.0023 N CH_3COOH, .0920 N CH_3COONa, PH 6.3, used with alizarin red as indicator.)

To 57.5 c.c. .1 N acetic acid add 46 c.c. .1 N sodic hydrate and dilute to 500 c.c.

SOLUTION 9. (.0046 N CH_3COOH, .0920 N CH_3COONa, PH 6.0, used with alizarin red as indicator.)

To 69 c.c. .1 N acetic acid add 46 c.c. .1 N sodic hydrate and dilute to 500 c.c.

SOLUTION 10. (.0092 N CH_3COOH, .0920 N CH_3COONa, PH 5.7, used with alizarin red as indicator.)

To 92 c.c. .1 N acetic acid add 46 c.c. .1 N sodic hydrate and dilute to 500 c.c.

SOLUTION 11. (.023 N CH_3COOH, .0920 N CH_3COONa, PH 5.3, used with alizarin red, or methyl red, as indicator.)

To 161 c.c. .1 N acetic acid add 46 c.c. .1 N sodic hydrate and dilute to 500 c.c.

SOLUTION 12. (.0460 N CH_3COOH, .0920 N CH_3COONa, PH 4.9, used with alizarin red, or methyl red, as indicator.)

To 55.1 c.c. .5 N acetic acid add 46 c.c. .1 N sodic hydrate and dilute to 500 c.c.

SOLUTION 13. (.0920 N CH_3COOH, .0920 N CH_3COONa, PH 4.7, used with alizarin red, or methyl red, as indicator.)

To 110 .2 c.c. .5 N acetic acid add 46 c.c. .1 N sodic hydrate and dilute to 500 c.c.

The determination is carried out as follows: Transfer 10 c.c. urine to a 250 c.c. Florence flask. A large number of exactly similar flasks (20-30) are indispensable for this determination. Transfer 10 c.c. of standard solution to each of a series of similar flasks, numbering each flask according to the standard solution it contains, then fill all the flasks to the neck with distilled water, and add the indicator (7.5 mg. alizarin red). Determine by in-

spection which standard solution most nearly matches the urine.

If the result obtained in the determination with alizarin red indicates an acidity greater than 5.3 (PH), the determination should be repeated, with .15 c.c. saturated solution of methyl red (in 50 per cent. alcohol), as indicator.

With urines having an acidity of 5.3-6.7 (PH) repeat the determination, as with alizarin red, but employing neutral red (15 mg.) as indicator.

With urines which are alkaline to phenolphthalein use phenolphthalein as indicator.

In the case of albuminous urines the original determination should be made with methyl red, instead of alizarin red, as indicator.

BLOOD

Determination of Non-protein Nitrogen in Blood (*J. Biol. Chem., 11, 527*).—To a 50 c.c. volumetric flask add 25-35 c.c. pure "acetone free" methyl alcohol. Then add with a pipet and with constant shaking 5 c.c. of blood. Fill to the mark with methyl alcohol, shake, and let stand for two hours. Filter on a dry filter paper; to the filtrate add two or three drops of saturated alcoholic solution of zinc chlorid (the latter must be free from ammonia). After standing for a few minutes filter again; a clear and perfectly colorless filtrate should be obtained.

Transfer 10 c.c. of the alcoholic filtrate to a large Jena test tube of the kind used in urine analysis. Add one drop of sulphuric acid, one of kerosene, and a pebble. Drive off the methyl alcohol by immersing the test tube in a beaker of boiling water for five to ten minutes. When the alcohol is removed, add 1 c.c. of concentrated sulphuric acid, a gram of potassium sulphate, and a drop of copper sulphate solution. Boil the mixture, cool, and dilute as in the analysis of urine (see p 85).

From this digestion mixture remove the ammonia in the usual manner, and collect it, not in a measuring flask (as in urine analysis), but in a second large test tube previously charged with 1 c.c. of .1 N acid and 2 to 3 c.c. of water. The reason for this variation is that 1 c.c. of blood contains usually not over .2-.3 mg. of non-protein nitrogen. The final Nesslerized solution, therefore, cannot be diluted to 100 c.c., and smaller volumetric flasks cannot be used as receivers during the air current treatment because of spattering. Large test tubes are therefore used as receivers, and the ammonia is Nesslerized in these before the liquids are transferred to measuring flasks. Ordinarily the colored solutions obtained are transferred to 10 c.c. flasks, and are then found to have a depth of color which permits of a sure and accurate reading in the colorimeter.

In all ordinary cases use 7 to 8 c.c. of diluted Nessler's reagent (dilution 1:5) for the production of the color. If much ammonia

is present, so that the resulting colored solution must be diluted to 50 c.c., correspondingly larger amounts of Nessler's reagent should be added.

Make the colorimetric comparison with a standard of 1 mg. ammonia nitrogen, obtained from ammonium sulphate and Nesslerized as in urine analysis (in 100 c.c. flasks).

Calculate the results to milligrams of non-protein nitrogen per 100 c.c. of blood.

CALCULATION: Multiply 20 (the colorimeter standard in m.m.) with 10 (or any other volume in c.c. to which the Nesslerized unknown has been diluted), and divide by R (the reading of the unknown in m.m.). The result will be found to correspond to the non-protein nitrogen in mg. per 100 c.c. of blood.

Before cancellation the formula is:

$$\underset{(1)}{\frac{20}{R}} \times \underset{(2)}{\frac{10}{100}} \times \underset{(3)}{\frac{100}{1}} = \text{mg. per 100 c.c.}$$

The first term (1) represents the colorimetric comparison, the second term (2) the relative dilutions of the Nesslerized solutions, and the third term (3) the volumes of blood involved.

Preparation of Nessler's Reagent.—Dissolve 200 g. of potassium hydroxid (or sodium hydroxid) in 950 c.c. of distilled water in a flask, and cool under running water. Transfer 40 c.c. of distilled water, 55 g. of potassium iodid, and 100 g. of mercuric iodid to a large heavy beaker (capacity 1500 c.c.) or a glass jar. Immerse the lower part of the beaker in warm water (40-50° C.), and shake gently until the last trace of the red iodid of mercury is dissolved. The solution occurs rapidly, and is usually finished in 10-15 minutes. Remove from the warm water, and pour the cooled hydroxid solution into the yellow iodid mixture in a slow but continuous stream and with vigorous stirring. The mixture should remain clear, or at the most slightly opalescent. If a moderate sediment has formed or should later form in the solution, the clear supernatant liquid is still good. Keep in well-stoppered bottles, preferably in the dark.

Determination of Urea in Blood (*J. Biol. Chem., 11, 531*).—Measure 10 c.c. of the alcoholic filtrate used for the determination of the non-protein nitrogen into one of the large Jena test tubes in which the decomposition of the urea is to be made. Add

a drop of dilute acetic acid, and two or three of kerosene, and close the test tube by a two-hole rubber stopper. (Through one of the holes in the stopper is passed a glass tube drawn out to a capillary several inches long. The capillary end reaches nearly to the bottom of the test tube. Through the other hole passes a short bent glass tube which is connected with a good water pump.) Place the test tube in warm water, and start the vacuum pump. In ten to thirty minutes the combined action of the gentle heat, the air current (through the capillary), and the vacuum, removes all the alcohol. Remove the rubber stopper, breaking off the capillary tube by bending it against the sides of the test tube. Add 2 c.c. of 25 per cent. acetic acid, a temperature indicator, a pebble, and 7 g. of dry potassium acetate; then heat to 153-158° C. for about eight to ten minutes, exactly as in the urea determination described for urine (see p. 87).

Collect the ammonia, set free by the subsequent air current treatment, in a large test tube, Nesslerize (usually with only 3 c.c. of the diluted reagent), make up to volume in a 10 c.c. volumetric flask, and make the color comparison, as in the case of the total non-protein nitrogen, against the same standard solution of ammonium sulphate.

Urease Method for the Determination of Urea in Blood (*Marshall, J. Biol. Chem., 15, 487*).—Prepare a dilute urease solution by mixing one volume of the urease extract used for the determination of urea in urine (p. 89) with nine volumes of water.

Blood seems to contain a substance ("auxo-urease") which greatly accelerates the action of soy bean urease (about eight times). (Neumann: *Bioch. Zeitschr., Vol. 69.)* Since all urease extracts contain traces of free ammonia, the use of unnecessarily large amounts of the ferment is to be avoided.

With a pipet containing a little powdered sodium citrate in the tip, measure 5 c.c. of blood with gentle shaking into a 25 c.c. volumetric flask half full of water. Fill to the mark with water, using a drop or two of ether to dispel the foam, and shake thoroughly.

Measure 5 c.c. of the diluted blood into a large test tube. Add 2 drops of kerosene and 1 c.c. of the diluted urease extract. Stopper with the aëration apparatus, and let stand 15 minutes. Attach the apparatus to an absorption tube, the latter reaching to the bottom of a large test tube containing 1 c.c. tenth normal

HCl and 2 c.c. of water. At the end of the 15 minutes pass the air current through the apparatus for a few seconds (to sweep out any ammonia which exists as a vapor). Stop the air, open the test tube, and add about 5 g. solid potassium carbonate. Close the tube, and aërate in the usual manner for 10 to 15 minutes.

To the contents in the receiver add .5 c.c. Nessler's reagent previously diluted with 2 to 3 volumes of water. Transfer the Nesslerized solution to a 10 c.c. volumetric flask (using less than 5 c.c. of water for washing), and fill up to the mark. Compare the color with that of the standard ammonium sulphate solution Nesslerized in the usual manner.

The Determination of Ammonia in Blood (*J. Biol. Chem., 11, 534*). —Reasonably accurate determinations of ammonia in blood are obtained with great difficulty because of the decomposition of certain nitrogenous components of blood even at room temperatures, and because the free ammonia actually present in fresh blood amounts only to a few hundredths of a milligram per 100 c.c.

Transfer 10 c.c. of blood to a large test tube. Add 2-3 c.c. of a solution containing 10 per cent. sodic carbonate and 15 per cent. potassium oxalate. Then aspirate the liberated ammonia by means of a rapid air current into a test tube containing 1 c.c. of water and 5-6 drops .1 N hydrochloric acid. Time 20-30 minutes. Nesslerize this solution by the gradual addition of not over 1 c.c. diluted Nessler's solution (dilution 1 : 5). Transfer the solution to a 10 c.c. volumetric flask, and dilute to volume with "ammonia free" water.

"Ammonia free" water is obtained from ordinary distilled water by the addition of a little bromin water and a few drops of concentrated sodium hydroxid.

The colorimetric valuation of the solution by means of the Duboscq colorimeter cannot be accomplished without materially altering the instrument. An iris diaphragm should be attached to one sliding platform of the colorimeter, so as to regulate the amount of light passing through on that side. The hexagonal prism should be removed from the opposite side. With these alterations the Nesslerized solution in a 100 m.m. polariscope tube may be compared with .5 or 1 mg. ammonia (Nesslerized and diluted to 100 c.c.). Place the standard in the cup on the side of the iris

diaphragm, fill a 100 m.m. polariscope tube with the unknown, and insert this on the other side. Adjust the standard until the two fields are equal.

Method for the Determination of Uric Acid in Human Blood *(Compare J. Biol. Chem., 13, 469 and 20, 629).*—Pipet 25 c.c. of distilled water into a 50 c.c. Erlenmeyer flask, and with a pipet add 5 c.c. of blood. Rinse the latter pipet once or twice with the diluted blood in the flask. Heat the mixture for 4-5 minutes by immersion in a beaker of boiling water, rotating occasionally so as to secure a uniform coagulation. Next add 1 c.c. of an acid sodic acetate solution (made by dissolving 1 g. glacial acetic acid and 12.5 g. crystallized sodic acetate in 10 c.c. of water), mix, and heat a moment more to complete the coagulation. Remove the flask from the water-bath, add 4 c.c. of diluted (2.5 per cent.) colloidal iron solution (equal parts of commercial colloidal iron and distilled water), and mix thoroughly. Filter through a small, dry, folded filter into a dry flask. At the end of the filtration press the filter paper containing the coagulum gently against the side of the funnel so as to obtain the maximum amount of filtrate.

Transfer 11 c.c. of the blood filtrate to a centrifuge tube, add 2 c.c. of acid silver lactate solution (silver lactate 5 per cent., lactic acid 5 per cent.), stir the mixture with a small glass rod, and rinse the rod with a few drops of distilled water. Centrifuge for 2-3 minutes and pour off the supernatant liquid. Then add 10 c.c. more of the blood filtrate to the same tube, and also another 2 c.c. of the silver lactate solution. After stirring and rinsing off the rod, centrifuge again for 2-3 minutes, and pour off the liquid.

The centrifuge tube now contains the precipitate from 21 c.c. of the blood filtrate. Since the coagulation mixture before filtration contained 25 c.c. of water, 5 c.c. of blood, 4 c.c. iron solution, and 1 c.c. of acetate solution (a volume of 35 c.c.), 21 c.c. of the filtrate represents 3 c.c. of blood.

Add to the precipitate in the centrifuge tube about 5 c.c. of water and 3 drops magnesia mixture, stir the precipitate with a rod, and add ammonia (1-2 c.c.) until the silver chlorid is completely dissolved and only the flocculent silver magnesium urate is left. Rinse the rod, and centrifuge the tube for about 2 minutes. Decant, add about 5 c.c. of water, then stir the precipitate thoroughly (again carefully rinsing the stirring rod), centrifuge (2 minutes) and pour off the liquid.

Add about 5 c.c. of water, .5 c.c. of 5 per cent. sodium cyanide solution (*poisonous: 3 c.c. may be fatal dose*), and 1 c.c. of uric acid (phenol) reagent, and stir. A perfectly clear solution should result. Add 5 c.c. of 20 per cent. sodium carbonate solution and allow the tube to stand for 20 minutes.

Measure 10 c.c. (.5 mg.) of standard uric acid solution and 50 c.c. water into a 100 c.c. graduated flask. Add 4 c.c. of uric acid (phenol) reagent, 2 c.c. of the sodium cyanide solution, and 20 c.c. of 20 per cent. sodium carbonate, and let the flask stand 20 minutes.

At the end of 20 minutes centrifuge for 2 minutes the tube containing the uric acid from blood. Pour the blue liquid as completely as possible into a 25 c.c. graduated flask, fill to the mark with water, and mix.

Fill the 100 c.c. flask containing the standard to the mark, shake, and centrifuge a portion.

Read these two solutions against each other in the colorimeter, setting the standard at 20 m.m.

In making the calculations it is to be noted that half a milligram of uric acid diluted to 100 c.c. is the standard, whereas the unknown represents 3 c.c. of blood diluted to 25 c.c. Twenty multiplied by 100, and divided by ($4 \times 2 \times 3$) and by the reading of the unknown, gives the uric acid in milligrams per 100 c.c. of blood.

Or: 833 divided by the reading of the unknown (in m.m.) gives the desired figure.

Preparation of Standard Uric Acid Solution.—In a small beaker weigh accurately 250 mg. of pure uric acid, and dissolve by the addition of 30-40 c.c. .4 per cent. solution of lithium carbonate. When solution is complete, transfer it without loss of a single drop to a 500 c.c. volumetric flask. Fill to the mark with distilled water and mix.

Transfer 100 c.c. of the freshly prepared uric acid solution to a liter flask, add 500-700 c.c. of water and 10 c.c. normal hydrochloric acid (or 1 c.c. concentrated acid), and fill up to the mark with water. Transfer the solution to a bottle, add 5 c.c. of chloroform, and mix.

The acidified uric acid solution keeps a long time (several weeks). From the fresh carbonate solution of uric acid several liters of the dilute acidified solution can of course be prepared, but it is not yet known (Oct., 1915) whether these solutions keep indefinitely.

Twenty c.c. of the solution contains 1 mg. of uric acid.

Preparation of Uric Acid and Phenol Reagent.—Transfer to a flask (capacity about 1500 c.c.):

750 c.c. of water,
100 g. of sodium tungstate,
20 g. of phosphomolybdic acid,
50 c.c. of phosphoric acid (85 per cent. H_3PO_4),
100 c.c. of concentrated hydrochloric acid.

Insert a funnel in the flask and partly close the opening of the funnel with a watch glass. Boil the mixture gently for two hours. A deep straw yellow solution should be obtained. It should not turn appreciably blue when a sample, 5 c.c., is rendered alkaline with sodic carbonate. Dilute to a liter.

Preparation of Uric Acid Reagent.—Introduce into a flask:

750 c.c. of water,
100 g. of sodium tungstate,
80 c.c. of phosphoric acid (85 per cent. H_3PO_4).

Partly close the mouth of the flask with a funnel and small watch glass and boil gently for two hours. Dilute to a liter.

Colorimetric Method for the Determination of Creatinin in Blood *(J. Biol. Chem., 17, 475)*.—Transfer 10 c.c. of blood (or milk) to a 50 c.c. shaking cylinder which can be closed with a glass stopper. Fill up to the 50 c.c. mark with saturated picric acid solution and shake a few times. Add about 1 g. dry picric acid to the mixture and continue the shaking for five minutes. Transfer the mixture to centrifuge tubes, centrifuge, and pour the supernatant fluid through a filter. This is the most economical process where but little blood is available. When this is not the case, the quantities taken may be doubled, and the filtration can then be made without the preliminary centrifuging process.

By the above treatment the protein materials are removed and the creatin and creatinin are obtained in the picric acid filtrate. The filtrate is at the same time practically a saturated picric acid solution. For the colorimetric determination of the preformed creatinin in the filtrate prepare a correspondingly dilute solution of creatinin in saturated picric acid solution. A solution containing .2 mg. of creatinin per 100 c.c. is suitable for this purpose. This can be prepared in a few moments by transferring 1 mg. of creatinin from the standard creatinin solution used for urine work (see p. 97) to a 500 c.c. volumetric flask, and then making up to volume with saturated picric acid solution. This solution

can be kept on hand, as the creatinin is not precipitated on account of the great dilution. Add the same amount of alkali to equal volumes of filtrate from the blood (or milk) and of the known picric acid solution. The color produced corresponds to the amount of creatinin present, provided that neither contains more than one and one-half times as much as the other. It is absolutely essential, however, that exactly the same amount of the 10 per cent. alkali should be added to each solution, because in saturated solutions of picric acid the alkali deepens the color even when no creatinin is present. The amount of alkali found to yield the most reliable results, when making creatinin determinations in this manner, is 5 c.c. of 10 per cent. sodium hydrate per 100 c.c. of picric acid solution.

When measuring out the alkali with an ordinary buret the simplest way to get the same amount of alkali for the unknown and the standard is to determine how many drops, as obtained from the buret, correspond to 5 c.c., and then to add one-fifth of that number to 20 c.c. of the unknown filtrate and to 20 c.c. of the known solution. Ten c.c. of the filtrate from the blood (or milk) may be used for the color comparison, in which case the alkali added must be only as many drops as correspond to .5 c.c.

Sometimes the filtrate obtained from blood becomes slightly turbid after the addition of the alkali. It must then be centrifuged or filtered before using it for the color comparison.

Ten minutes' standing after the addition of the alkali is adequate for the development of the color, and the solutions are then ready for the color comparison without any further dilution. They are accordingly transferred to the cylinders of the Duboscq colorimeter and compared in the usual manner. The standard creatinin solution in this case can advantageously be set at 20 mm. because the color of the solutions are not very deep, but it is not at all essential that this should be done.

The calculation of the creatinin in the blood (or milk) is the same whether the standard is set at 10, 15, or 20 mm., and whether 10 or 20 c.c. of the filtrate was taken for the making of the color reaction. When 10 c.c. of blood is diluted to 50 c.c., or 20 c.c. to 100 c.c., and the standard contains .2 mg. of creatinin per 100 c.c., according to the directions described above, the reading of the standard divided by the reading of the unknown gives, without any further calculations, the creatinin in milligrams contained in 100 c.c. of blood (or milk).

Colorimetric Method for the Determination of Creatin plus Creatinin in Blood (Milk).—For the determination of the so-called total creatinin in blood, milk, and exudates, the preliminary precipitation with picric acid is conducted in exactly the same manner as has been described above in connection with the determination of the preformed creatinin. The filtrate obtained from 10 c.c. of blood diluted with picric acid solution to 50 c.c. amounts usually to rather more than 30 c.c., and 10 c.c. of this filtrate is all that is needed for the total creatinin determination. Measure out 10 c.c. of the creatin-creatinin filtrate into a small Erlenmeyer flask (capacity 25 or 50 c.c.) or large test tube.

Cover the flask or test tube containing the filtrate with tinfoil, transfer to the autoclave, and heat to about 120° C for about twenty minutes. When using the autoclave, it is important not to open it at the end of the heating until the temperature has fallen to below 100° so as to avoid all mechanical losses.

When cooled to room temperature, rinse the solution into a 25 c.c. volumetric flask, or a measuring cylinder, with saturated picric acid solution, making a volume of 25 c.c. Add 1.25 c.c. of 10 per cent. sodium hydrate solution for the development of the color reaction.

Two standard creatinin solutions in saturated picric acid are necessary in this determination because of the variations in the creatin contents of normal blood. When working on hospital patients, the variations are greater still, and three standard creatinin determinations are desirable. These standard solutions contain .5, 1, and 2 mg. of creatinin, respectively, per 100 c.c. of saturated picric acid solution.* To 20 c.c. of each of these solutions in measuring cylinders add 1 c.c. of 10 per cent. sodium hydrate. By inspection one can readily tell which standard comes nearest to having the same color as the unknown, and with this as a standard make the color comparison in the usual manner, by the help of the Duboscq colorimeter. The colors of these solutions are much deeper than those obtained in the determination of the preformed creatinin, and the standard solution is consequently usually set at 10 mm.

The reading of the standard in mm., multiplied by 125 and by .5, 1, and 2, according to which standard is used, when divided

* These solutions are made by putting 1, 2, and 4 c.c. of the standard creatinin zinc chlorid solution in 200 c.c. volumetric flasks, and making up to volume with saturated picric acid solution.

by the reading of the unknown in mm., gives the amount of creatin-creatinin in milligrams per 100 c.c. of blood (or milk).

Determination of Chlorids in Blood, Exudates, and Transudates (*McLean and Van Slyke: J. Biol. Chem., 21, 361* [*1915*]).—In this method the chlorids are precipitated in the presence of nitric acid by standard silver nitrate solution, the silver chlorid is removed by filtration, and the excess of silver is titrated back with standard potassium iodid solution. The titration is performed in the presence of nitrous acid and starch, so that the first drop of iodid in excess of the silver present is changed to free iodin and gives the blue starch-iodin color. The optimum acidity for the end-point is fixed by the addition of trisodium citrate in amount equivalent (⅓ molecule) to the free nitric acid present.

The solutions required are

I. An acid solution of silver nitrate, 1 c.c. of which is equivalent to 2 mg. of NaCl. This solution contains 5.812 g. silver nitrate and 250 c.c. concentrated nitric acid (sp. gr. 1.42) in one liter.

II. A solution of potassium iodid, 1 c.c. of which is equivalent to 1 mg. of NaCl. This solution contains 3 g. of potassium iodid per liter.

III. A solution containing sodium citrate, sodium nitrite, and starch, which substances respectively regulate the acidity, provide an oxidizing agent for the iodid, and serve as indicator. This solution contains 446 g. of trisodium citrate, 20 g. of sodium nitrite, and 2.5 g. of soluble starch per liter.

First dissolve the starch in about 500 c.c. of boiling water. Add the citrate and nitrite, and heat the mixture until all is dissolved. Filter the solution, while still hot, through cotton, wash the filter with hot water, cool, and finally make up to a volume of 1,000 c.c.

Standardize solution II. against solution I. by adding 5 c.c. of the latter to 5 c.c. of solution III., and titrating with solution II. until the blue end-point is obtained. On the basis of this titration, dilute the iodid solution to such a degree that 10 c.c. is exactly equivalent to 5 c.c. of the silver solution.

As a preliminary step to the titration of chlorids in blood, or other albuminous fluids, the protein must first be removed. This is accomplished as follows: Run 2 c.c. of oxalated blood from an Ostwald pipet into 10 c.c. of 10 per cent. magnesium sulphate solution contained in a 20 c.c. volumetric flask. Rinse the pipet

twice by drawing up into it the solution from the flask. Add 2 drops of 50 per cent. acetic acid, fill to the mark with water, invert a few times to secure a thorough mixing of its contents, and finally place for 10 minutes in a beaker of boiling water. After cooling pour the contents of the flask onto about .3 g. of Merck's blood charcoal in a small beaker. Let stand a few minutes and filter. A water clear filtrate should be obtained, which is now ready for titration.

The final titration of the protein free filtrate is carried out as follows: Pipet 10 c.c. of filtrate (equivalent to 1 c.c. of blood) into a 25 c.c. volumetric flask, add 5 c.c. of the silver nitrate solution, and fill to the mark with distilled water. Mix, allow to stand five minutes, and filter through a dry filter paper. Pipet 20 c.c. of this filtrate into a 50 c.c. Erlenmeyer flask, add 4 c.c. of solution III., and then run in the potassium iodid (solution II.) from a buret until the end-point is reached. The first definite blue color marks the end-point, and with practice is easily determined. The results may be calculated from the following formula when 20 c.c. of filtrate from the silver chlorid is titrated:

$$\text{Grams NaCl per liter} = \frac{12.5\ (8 - \text{c.c. KI solution used})}{\text{c.c. filtrate (from charcoal) used}}$$

Before beginning work it is desirable to make sure by qualitative tests that the chemicals employed (more particularly the magnesium sulphate, potassium nitrite, and charcoal) are free from chlorids. Before making up solution III it is also well to determine whether the soluble starch provided gives a *blue* end-point with iodin. Much of the soluble starch on the market is so highly dextrinized that it gives a reddish-brown color with iodin and is therefore unfit for titration work.

Nephelometric Method for the Determination of Fat in Blood (*Bloor: J. Biol. Chem., 17, 377*).—Run 3 c.c. of blood slowly and with shaking into a 100 c.c. volumetric flask containing about 80 c.c. of a mixture of redistilled alcohol and ether (3:1). Raise the contents of the flask just to boiling (with constant shaking) in a water-bath, cool in running water, make up to the 100 c.c. mark with more alcohol-ether, mix, and filter into a small flask or bottle. Stopper tightly as soon as filtration is finished to avoid loss of liquid by evaporation.

Measure 15 c.c. of the filtrate, containing about 2 mg. of fat, into a small beaker, add 2 c.c. of N sodium ethylate, and evaporate the mixture just to dryness on the water-bath. To the dry residue add 5 c.c. of alcohol-ether (1:3), and warm gently until the flakes of alkali are loosened from the bottom of the beaker. To the mixture add 50 c.c. of water, and stir until a clear solution is obtained. Add 5 c.c. of a standard solution of oleic acid in alcohol-ether, containing about 2 mg. oleic acid, to 50 c.c. of water in another beaker. To the standard, and to the blood fat solution add (as nearly simultaneously as possible) 10 c.c. of 10 per cent. hydrochloric acid. Allow the suspensions so produced to stand for five minutes, and then compare by means of the Duboscq colorimeter previously converted into a nephelometer.

For the comparison, fill the two nephelometer tubes, after rinsing with the solutions, to the same height (the meniscus slightly above the dark collar at the top of the tubes), and place in the nephelometer, with the standard tube always on the same side. Set the movable jacket on the standard tube at a convenient point (30 mm. in the modified colorimeter described below), and make comparisons by adjusting the jacket on the test solution until the images show equal illumination. Make five readings alternately from above and from below, and take the average as the reading. Make the calculations in the same way as in the colorimetric methods, the values being inversely proportional to the readings.

Changing the Duboscq Colorimeter into a Nephelometer (*J. Biol. Chem., 22, 145*).—A simple method for transforming the Duboscq colorimeter into a nephelometer is described in the *Journal of Biological Chemistry,* Vol. 22, p. 145, 1915. The extra parts necessary are supplied in an improved form by the International Instrument Company of Cambridge, Mass. By the use of these parts the change may be quickly made as follows: Unscrew the movable glass prisms of the colorimeter, slip the brass collars for the nephelometer tubes into place, and fasten on the plate from which the prisms were removed. Slip the movable jackets into the holes in the cup supports, and after pushing the nephelometer tubes into place in the collars, the instrument is ready for use. A darkened room and a light-tight box for the light are necessary. The box should be about 48 cm. long, 32 cm. high, and 20 cm. wide for the ordinary colorimeter. It should contain

a bracket at one end to support the light (a 50 watt "Mazda") at the height of the nephelometer tubes, and a stop at the other end, against which the instrument may be pushed and so placed that the nephelometer tubes are about 30 cm. from the light. A slot in the top of the box to receive the telescope of the instrument and a dark curtain to cover the end of the box after the instrument is pushed into place complete the equipment of the box. All exposed parts should be painted a dull black.

Since the readings obtained from suspensions of different strength are not exactly proportional to the amount of precipitate present, it is necessary to calibrate the instrument for different strengths and make corrections accordingly. If, however, the solution to be tested is within 25 per cent. of the value of the standard, no correction is necessary.

A Method for the Determination of Cholesterin in Blood or Blood Serum (*Bloor, not yet published*).—The method consists in the application of the Autenrieth-Funk procedure (Autenrieth and Funk—*Münch. med. Wochenschr., 1913, Vol. 69, p. 1243)* to the alcohol-ether extract of blood or serum prepared as for the determination of fat.

Measure 10 c.c. of the extract into a small beaker, and evaporate *just* to dryness on the water-bath or electric stove. (Any heating after dryness is reached produces a brownish color, which makes the determination difficult or impossible.)

Extract the cholesterin from the dry residue by boiling out 3 or 4 times with small portions (2-3 c.c.) of chloroform and decanting. Evaporate the combined extracts to a little less than 5 c.c., transfer to a 10 c.c. graduated cylinder, and make the volume up to 5 c.c. A little turbidity does not matter, since it disappears on adding the reagents. Measure 5 c.c. of a standard cholesterin solution in chloroform, containing .5 mg. of cholesterin, into a similar 10 c.c. graduate. Add to each 2 c.c. of acetic anhydrid and .1 c.c. of concentrated H_2SO_4. Mix the solutions by inverting two or three times, and set the cylinders in the dark for 15 minutes; then transfer the solutions to the colorimeter cups, and compare as usual, setting the standard at 15 m.m.

The cement of the colorimeter cups must, of course, not be soluble in chloroform. Plaster-of-Paris has been found satisfactory, or even ordinary glue, if the cups are not used for any other purpose.

Method for the Determination of Sugar in Blood (*Lewis and Benedict, J. Biol. Chem., 20, 61, 1915*).—Pipet 2 c.c. of oxalated blood into a 25 c.c. volumetric flask containing 5 c.c. of distilled water; the water and blood are mixed in order to insure complete hemolysis of the latter. Add 15 c.c. of saturated picric acid solution, fill up to the mark with water, and shake.

Filter, and measure two 8 c.c. portions of the filtrate into large Jena test tubes (22 × 120 m.m.) for duplicate determinations. Add 2 c.c. of saturated picric acid solution and exactly 1 c.c. of 10 per cent. sodium carbonate solution (as well as two glass beads or pebbles, and two or three drops of kerosene). Evaporate the contents of the test tubes rapidly over a free flame until precipitation occurs. Add about 3 c.c. of water, and heat the tube again to boiling so as to dissolve the precipitate. Now transfer the contents of the tube quantitatively to a 10 c.c. volumetric flask, cool, fill up to the mark with water, shake, then filter through cotton into the colorimeter chamber. Compare the color at once with that obtained from .64 mg. of dextrose, 5 c.c. of saturated picric acid, and 1 c.c. of saturated sodium carbonate, after the solution of these substances has been evaporated over a free flame and diluted to 10 c.c. in the same way as in the case of the unknown.

Instead of the dextrose standard a permanent standard may be used which consists of .064 mg. picramic acid and .1 g. sodium carbonate (anhydrous) in 1,000 c.c. Before being used this solution should be standardized against pure dextrose.

To calculate the results the following formula may be used:

$$\text{Mg. dextrose per c.c. of blood} = \frac{\text{reading of standard}}{\text{reading of unknown}} \times \frac{\text{mg. dextrose in standard}}{\text{c.c. of blood used}}$$

But as the amount of dextrose in the standard is .64 mg. and the amount of blood used is .64 c.c., the second fraction equals unity, and the equation is:

$$\text{Mg. dextrose per c.c. of blood} = \frac{\text{reading of standard}}{\text{reading of unknown}}$$

The per cent. of blood sugar is, of course, one tenth of the figure thus obtained.

THE END

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